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SECTION MEETINGS

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| **CLEVELAND, O.** | |
| Western Reserve University | April 8, 1949<br>May 13, 1949 |
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| Michael Reese Hospital, Chicago | May 10, 1949 |
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| **WISCONSIN** | |
| University of Wisconsin | April 28, 1949 |

## 17120. Proteolytic Enzyme Inhibitors of Human Serum in Health and Disease.*†

P. M. West and J. Hilliard.‡ (Introduced by A. White.)

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The antiproteolytic power of blood serum has been described in the reports of Flexner *et al.*,[1] Sure,[2] Weil and Russell[3] and Clark *et al.*[4] Following purification of several proteolytic enzymes it became possible to determine the probable nature of this enzyme antagonism. Crystalline pepsin inhibitor isolated by Herriott[5] and trypsin inhibitor by

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† Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

‡ The authors wish to express their gratitude to Betty Smith and Willard Keye for their excellent technical assistance and to the following physicians who supplied valuable clinical material: C. K. Emery, H. C. Weaver, A. C. Mietus, and G. J. Hummer.

[1] Flexner, L. B., Berkson, J., Winters, H., and Wolman, I., Proc. Soc. Exp. Biol. and Med., 1929, **26**, 592.

[2] Sure, B., *Biochem. J.*, 1935, **29**, 1508.

[3] Weil, L., and Russell, M. A., *J. Biol. Chem.*, 1938, **126**, 245.

[4] Clark, D. G. C., Clifton, E. E., and Newton, B. L., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 276.

[5] Herriott, R. M., *J. Gen. Physiol.*, 1941, **24**, 325.

Kunitz and Northrop[6] have been shown to be highly specific, reacting to form inert dissociable complexes with the respective proteinase. As a sensitive means of studying protein metabolism in various pathological states, an investigation of the role of certain of the circulating proteolytic enzyme inhibitors appears to offer a new approach of considerable promise. This paper presents a method for quantitative estimation of both rennin and chymotrypsin inhibitors of serum, together with results obtained in a survey of 2000 patients.

*Materials and methods.* The method is based on the milk coagulating property of both chymotrypsin and rennin. Determinations are made on fresh serum or serum stored at ice-box temperature not over 24 hours. Fasting blood is not required. 1.*Enzyme Standardization.* (a) *Chymotrypsin.* Two to 3 mg crystalline chymotrypsin (supplied through courtesy of Spicer-Gerhart Company, Pasadena, California) are dissolved in 10 ml of distilled water. 0.2 ml of solution is pipetted into two 13 x 120 mm tubes containing 2.0 ml of fresh homogenized milk (pH 6.6) which is at ice-box temperature. A stop watch is started, contents of tubes mixed quickly by rotation, and placed immediately in a water bath at 37° ± 0.1°. If coagulation has not occurred in 9 minutes tubes are shaken gently every 15 seconds. The time at which the first granular precipitation of calcium paracaseinate appears in the film on the wall of the tube is the end point. Dilution of enzyme solution is continued until the end point occurs at 10 minutes 45 seconds to 10 minutes 50 seconds. This precise time is selected to provide reasonable stability of the enzyme as well as optimum conditions for measurement of inhibitor. At 10 minutes, variation in inhibitor levels is barely detectable, and over 11 minutes, inhibition is too great for accurate measurement and the enzyme is unstable. It is essential that the enzyme activity fall exactly within the 5 second time limitation. The enzyme will retain this activity for about 3 hours. Stability is not improved by buffering. No variation attributable to natural homogenized milk as a substrate has been encountered in over a year of continuous use of the method. Powdered and condensed varieties are unsuited to accurate detection of the end point. Both milk and enzyme are kept in the ice-box except when actually in use. (b) *Rennin.* One rennet enzyme tablet ("Junket" brand, Chr. Hansen's Laboratory Inc., Little Falls, N. Y.) is dissolved in 80 ml of distilled water, and filtered after 10 minutes. Activity is adjusted as for chymotrypsin to an end point of 10′ 55″ to 11′ 0″. The difference of 10 seconds between the two enzymes allows measurement of inhibitors over a comparable range. 2. *Determination of Inhibitor.* (a) *Chymotrypsin inhibitor.* Two control tubes and 6 others representing 3 duplicate serum determinations are set up together. Dilute serum 1:10 with distilled water and pipette 0.04 ml into each tube. 2 ml of milk is added and the contents mixed. Finally, 0.2 ml of standardized chymotrypsin solution is added quickly and accurately, a stop watch started, the eight tubes thoroughly mixed in 20 seconds or less, and placed together in the water bath. At 10′45″ when controls reach the end point the watch is reset. Units of inhibitor are the number of additional minutes required for the serum-containing tubes to reach the end point. Results must agree within 30 seconds. Failure to reach the end point 30 minutes beyond the control is equivalent to complete inactivation of enzyme and is therefore the maximum reading. Time units are not related arithmetically to actual units but this correction is not applied. (b) *Rennin inhibitor.* Proceed as above except that 0.05 ml of undiluted serum is required in this reaction and the maximum value obtainable is 50 units. 3. *Interpretation.* (a) *Chymotrypsin inhibitor*: normal range 5-10 units; 13-30 units abnormal. (b) *Rennin inhibitor*: normal ranges 5-10 (70%) and 20-50 units (30%); 13-18 units abnormal.

*Results.* In this preliminary survey, 2000 random blood specimens representing all available types of pathology were studied. Six to 8 weeks later, pertinent clinical and laboratory findings that had been collected on each case were assembled with special reference to the

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[6] Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

TABLE I.
Occurrence of Abnormal Levels of Chymotrypsin and Rennin Inhibitors in Various Groups of Patients.

| | No. of cases | Abnormal chymo and/or rennin | |
|---|---|---|---|
| | | No. | % |
| 1. Pregnancy (all stages) | 103 | 88 | 85.3 |
| 2. Malignancy (entire group) | 220 | 130 | 59.0 |
| ″ (active, untreated) | 133 | 118 | 88.7 |
| ″ (treated) | 87 | 12 | 13.7 |
| 3. Allergy | 33 | 9 | 27.2 |
| 4. Acute infections, active phase (bacterial and virus) | 176 | 45 | 25.5 |
| 5. Benign breast lesions | 33 | 8 | 24.2 |
| 6. Cardiovascular (entire group) | 101 | 20 | 19.8 |
| ″ (decompensation) | 23 | 12 | 52.1 |
| ″ (hypertension) | 22 | 8 | 36.3 |
| 7. Chronic infections (tuberculosis, syphilis, coccidiomycosis, bronchiectasis, etc.) | 140 | 26 | 18.5 |
| 8. Psychoneuroses and psychoses | 55 | 10 | 18.1 |
| 9. Traumatic damage and surgical procedures (within 1 week) | 280 | 45 | 16.0 |
| 10. Peptic ulcer, ileitis, colitis | 83 | 6 | 7.2 |
| 11. Metabolic (diabetes, cirrhosis, nephritis, arthritis, hyperthyroidism) | 137 | 7 | 5.1 |
| 12. Benign tumors (polyps, papilloma, fibromyoma, fibroma, lipoma, adenoma) | 75 | 4 | 5.3 |
| 13. Unclassified | 346 | 13 | 3.7 |
| 14. Healthy subjects (including routine pre-operative hernia, hemorrhoids, varicose veins, etc.) | 218 | 0 | 0.0 |
| Total | 2000 | 411 | 20.5 |

TABLE II.
Pathological and Physiological States Influencing Chymotrypsin and Rennin Inhibitors.

| | |
|---|---|
| A. Pathology influencing both chymotrypsin and rennin inhibitors with equal frequency. | |
| 1. Pregnancy. | |
| 2. Malignancy | |
| 3. Trauma and surgery | |
| 4. Cardiac decompensation | |
| 5. Chronic infections | |
| B. Pathology influencing chymotrypsin inhibitor predominantly. | |
| 1. Acute infections (bacterial and virus) | 24% of cases |
| C. Pathology influencing rennin inhibitor predominantly. | |
| 1. Hypertension | 31.8% |
| 2. Benign breast lesions | 24.2 |
| 3. Allergy | 21.2 |
| 4. Psychoneuroses | 18.1 |
| 5. Fractures (healing stage) | 17.5 |

exact condition of the patient at the time the inhibitor determination was made. Results were correlated with these facts and the conclusions are summarized in Tables I and II.

*Discussion.* From a survey of data presented, it seems probable that rennin and chymotrypsin inhibitors reflect different phases of metabolism, since the concentration of one is seen to vary independently of the other. However, in disturbances of fluid balance, both may follow the same trend. With diminished fluid output in persistent vomiting, burns, hemorrhage, shock, high fever or congestive failure, both values frequently increase together. That the full explanation may be more complex is suggested by the fact that most of the above conditions are characterized by increased excretion of adrenal cortical steroids having a profound influence on protein metabolism. Factors which influence the rennin inhibitor predominantly further implicate the steroid hormones. The association of estrogens with certain benign breast hyperplasias is well known and the significance of stimulated estrogenic or androgenic activity in healing fractures has been demonstrated by Albright and co-workers.[7] Rennin inhibitor may increase also in many of the psychoses which have been shown by Pincus and Elmadjian[8] to exhibit diminished adrenal response to stress. The unexpected finding that a high percentage of the hypertensive and allergic groups have abnormal rennin inhibitor levels is of interest but difficult to interpret. Studies to determine the direct effects of steroid hormone administration on the proteolytic enzyme inhibitors are being undertaken.

Pregnancy is usually accompanied by a pronounced elevation of both inhibitors in the mother's circulation. In contrast, the cord blood of the newborn is high only in rennin inhibitor, while the other is very low.

A study of malignancy by this method reveals a characteristic but fluctuating picture, the host doing well clinically when the rennin inhibitor only is elevated, as occurs early, or at intervals in the course of the disease, but failing rapidly when the chymotrypsin inhibitor rises very high and rennin inhibitor drops to low levels (unpublished data). Obviously, it would be impossible to diagnose malignancy by these methods since an increase in inhibitor of either proteolytic enzyme is not specific for cancer. The inhibitor concentrations are profoundly influenced by radiation therapy and surgical excision, and thus a low percentage of abnormal inhibitor levels was noted in treated cases. Repeated observations on the serum inhibitors in cases with neoplasm may prove to be of considerable prognostic value and may indicate when further treatment is required as well as the effectiveness of the treatment given. This appears to us to be the chief value of the method as applied to the cancer patient. This aspect of the problem is being extended at the present time.

*Summary.* Methods are described for the determination of chymotrypsin and rennin inhibitors in serum. The many physiological and pathological conditions which influence their concentration are discussed.

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[7] Albright, F., Bloomberg, E., and Smith, P. H., *Trans. Assn. Am. Phys.*, 1940, **55**, 298.

[8] Pincus, G., and Elmadjian, F., *J. Clin. Endocrinol.*, 1946, **6**, 285.

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## 17121. A Comparison of Total Body Water as Determined by Antipyrine and Desiccation in Rabbits.

ROBERT J. SOBERMAN. (Introduced by Joel Warren.)

*From the Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.*

A method for determining total body water which employs the volume of uniform distribution of antipyrine, an analgesic drug, has been described recently.[1] Further support for the antipyrine method is presented in the present paper which records the values for total body water estimated by the antipyrine procedure and the values obtained by actual desiccation of the experimental animals.

*Experimental.* Four rabbits were injected intravenously with 60-240 mg of antipyrine according to body weight (approximately 100 mg/kg). Blood samples were drawn from each animal at 50, 75 and 100 minutes following the administration of the drug. The zero time plasma concentration (the concentration at the time of injection if uniform distribution

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[1] Soberman, R. J., Brodie, B., Hollander, V., Levy, B., Axelrod, J., and Steele, J. M., *J. Biol. Chem.*, 1949, **179**, 31.

TABLE I.
Comparison of Total Body Water in Rabbits Determined by Antipyrine and Desiccation.

| | | Total water | | | | |
|---|---|---|---|---|---|---|
| | | Antipyrine | | Desiccation | | |
| Rabbit | Weight (g) | (cc) | (% body wt) | (cc) | (% body wt) | Difference in (%) |
| 1 | 1782 | 1330 | 74.6 | 1350 | 75.8 | —1.2 |
| 2 | 1846 | 1270 | 68.8 | 1285 | 69.6 | —0.8 |
| 3 | 660 | 501 | 75.8 | 481 | 72.9 | +2.9 |
| 4 | 1172 | 912 | 77.8 | 902 | 77.0 | +0.8 |

TABLE II.
Antipyrine in Tissues of Rabbit 50 Minutes After Intravenous Injection.

| Tissue | Water (%) | Antipyrine in wet tissue ($\gamma$/g) | Antipyrine in tissue water ($\gamma$/ml) | Tissue water antipyrine / Plasma water antipyrine |
|---|---|---|---|---|
| Plasma | 92.0 | 39.6 | 43.1 | 1.00 |
| Heart | 73.7 | 30.5 | 41.3 | .96 |
| Liver | 72.0 | 33.2 | 46.1 | 1.08 |
| Muscle | 73.0 | 31.2 | 42.8 | .99 |
| Kidney | 78.6 | 33.1 | 42.1 | .97 |
| Lung | 71.6 | 31.1 | 43.5 | 1.01 |

was instantaneous and if none of the substance had been metabolized) is calculated by plotting the plasma levels on semilogarithmic paper as a function of time and extrapolating the straight portion of the time concentration curve (50, 75 and 100 minutes) to the time of injection. The value for total body water is obtained by dividing the amount administered by the plasma water level at zero time.

$$\text{Body water (liters)} = \frac{\text{Amt of drug injected (mg)}^{1}}{\text{Plasma water level (mg/L)}}$$

After the last blood sample had been drawn, the animals were weighed and sacrificed. The total animals were ground, and the final mass was then spread over large evaporating dishes and placed in a vacuum desiccator at a temperature of 85°C for a period of 5-7 days until constant weight had been achieved.

Since the rabbit, unlike a human, degrades antipyrine at a rapid rate (approximately 40% an hour), it appeared possible that the falling blood level indicated plasma degradation and not the concentration of antipyrine in the entire body. Accordingly, the distribution of antipyrine in the tissues of a fifth rabbit at the end of 50 minutes was determined. The water content of the tissues was determined by drying to constant weight at 100°C. The concentration of antipyrine in the tissues was measured and then calculated in terms of tissue water. The antipyrine is estimated directly in the plasma filtrate after deproteinization with zinc hydroxide. Sodium nitrite is added and the resulting 4-nitroso antipyrine measured in a spectrophotometer at 350 mu.[2]

*Results.* Comparison of the values obtained for total body water by antipyrine method and desiccation indicates close agreement between the two methods, thus confirming that antipyrine can be used as a measure of total body water (Table I).

The data of Table II demonstrate that the ratio of concentration of antipyrine in the tissues to that of plasma is one. Thus, the plasma concentration reflects the level of antipyrine in the entire body.

*Summary.* The close agreement obtained for the values of total body water by the use of the antipyrine method and desiccation, indicates the validity of this procedure for the purpose of measuring total body water.

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[2] Brodie, B., Axelrod, J., Soberman, R. J., and Levy, B., *J. Biol. Chem.*, 1949, **179**, 25.

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## 17122. Arterial Hypertension Produced by Experimental Stenosis of the Thoracic Aorta.

W. C. Sealy. (Introduced by J. W. Beard.)

*From the Department of Surgery, Division of Thoracic Surgery, Duke University School of Medicine, Durham, N. C.*

Arterial hypertension has been produced in dogs by stenosis of the thoracic aorta. The hypertension is sustained and has many of the characteristics of that observed in coarctation of the aorta in man.

*Materials and methods.* The method of producing permanent and prolonged stenosis of the thoracic aorta in dogs, described in detail elsewhere,[1] consists in the substitution of a section of lucite tubing for a segment of the aorta. This tube is designed to reduce turbulence by making the bore hourglass in shape. In the dogs used in this experiment, the tube was 3.7 cm long with 3 mm as the narrowest diameter. The lumen of the aorta was reduced in the dogs in this group approximately 75 to 85%.

Blood pressure records were obtained by direct puncture of the femoral or carotid arteries and measurement of the pressure with either a mercury or Hamilton manometer. From the records obtained with the Hamilton manometer, the systolic and diastolic pressures were calculated. Mean pressures were obtained either from estimations with the planimeter of the area under the pulse waves, or read directly from the mercury manometer. All blood pressure determinations were made with the animals under nembutal anesthesia, 27 mg per kg of body weight.

*Results.* There were 11 of 16 dogs that survived the operative procedure. Six of the 11 died at intervals of 7 days to 3 weeks after operation, 4 dying because of spastic paralysis of the lower part of the body. Of the 5 animals that survived for longer than one month, 4 are still living. One dog died at the end of 4 months from unknown cause. The remainder are still living at intervals of 4 to 14 months after stenosis of the aorta.

The changes in the blood pressures in these animals has followed, in all instances, a definite pattern as demonstrated in Table I. The femoral mean pressure has shown a drop after operation that persisted in some animals for as long as 7 days; but by the 14th day the pressure returned to normal. After 2 weeks the femoral mean pressure increased above the normal. The carotid mean pressure increased by the end of the first week to levels well above normal, and by 14 days reached hypertensive levels. The mean pressures in both the femoral and carotid increased during the next 3 months. After this time there was in the 2 dogs observed for one year, a decrease in the mean pressure both above and below the constriction, but the pressure still remained in the hypertensive range. The results with 5 animals are tabulated in Table II.

Recordings of the blood pressure with the Hamilton manometer have shown a large increase in the pulse pressure above the constriction and a decrease below. The diastolic pressures both above and below the constriction have increased along with the rise in the mean pressure.

*Discussion.* The experiments described here show that severe arterial hypertension can be produced in dogs by stenosis of the thoracic aorta. The various mechanisms that contribute to the elevation of the blood pressure are not evident from these results. It has been suggested that the hypertension in coarctation of the aorta is renal in origin.[2] Though the blood flow to the kidneys is not recorded in these experiments, it is interesting to note that for only about 14 days was the mean pressure below the constriction lower than the normal. After this interval there was a progressive rise in the mean pressure. On the other hand, there was a diminution in the

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[1] Sealy, W. C., and McSwain, George H., *Surgery*, in press.

[2] Rytand, D. A., *J. Clin. Invest.*, 1938, **17**, 391.

TABLE I.
Experimental Hypertension. Blood pressure changes associated with stenosis of the thoracic aorta in the dog.

| Days after constriction | Blood pressure in mm mercury Femoral Mean | S/D | Blood pressure in mm mercury Carotid Mean | S/D |
|---|---|---|---|---|
| Normal | 127 | 165/108 | 126 | 149/110 |
| 6 | 45 | 45/45 | 138 | 161/116 |
| 16 | 133 | 151/121 | 158 | 200/137 |
| 30 | 157 | 181/135 | 190 | 233/149 |
| 40 | 169 | 184/154 | 197 | 236/162 |
| 124 | 176 | 199/167 | 238 | 292/198 |

S = Systolic. D = Diastolic.

TABLE II.
The Blood Pressure Changes Associated with Experimental Coarctation of the Aorta in Dogs.

| Animal No. | Normal blood pressure Femoral M | S/D | Carotid M | S/D | Duration of aortic constriction, mo. | Last recorded blood pressure Femoral M | S/D | Carotid M | S/D |
|---|---|---|---|---|---|---|---|---|---|
| 14 | 118 | | 124 | | 14 | 133 | 144/123 | 158 | 185/130 |
| 16 | 128 | | 130 | | 5 | 196 | | 222 | |
| 22 | 134 | | 140 | | 13 | 154 | 170/137 | 193 | 241/160 |
| 29 | 127 | 165/108 | 126 | 149/110 | 4 | 176 | 199/167 | 238 | 292/198 |
| 31 | 125 | 157/107 | 161 | 184/146 | 3 | 162 | 170/157 | 239 | 299/199 |

M = Mean pressure. S = Systolic. D = Diastolic.

pulse pressures below the constriction that persisted. This might be interpreted as evidence for renal origin of the hypertension as suggested by Kohlstaedt and Page.[3]

The substitution of a rigid narrow tube for a section of the aorta would alter the characteristics of the pulse waves above and below the constriction; but this would not explain the marked elevation of the blood pressure that occurs in experimental stenosis of the aorta.

*Summary.* 1. By stenosis of the thoracic aorta in dogs, arterial hypertension can be produced above and below the stenosis.

2. This type of experimental hypertension has many of the characteristics observed in the hypertension occurring in coarctation of the aorta in man.

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[3] Kohlstaedt, K. G., and Page, I. H., *J. Exp. Med.*, 1940, **72**, 201.

# 17123. The Blood Volume of the Adult Rat, as Determined by $Fe^{59}$ and $P^{32}$ Labelled Red Cells.*

Nathaniel I. Berlin,[†] Rex L. Huff,[‡] Donald C. Van Dyke,[§] and Thomas G. Hennessy. (Introduced by John H. Lawrence.)

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The measurement of the blood volume of the rat by the use of plasma diluents has been reported by several investigators,[1-6] but there have been no reports on the use of labelled red blood cells for this purpose.

$Fe^{59}$ given enterally or parenterally has been shown by Hahn and co-workers[7] to be incorporated into the red blood cell as an integral part of the hemoglobin molecule. The plasma iron does not exchange with the iron incorporated into the hemoglobin.[8] Hevesy and Zerahn[9] have modified the Hahn and Hevesy[10] method for determining the total red cell volume by using red blood cells labelled by the incorporation *in vitro* of $P^{32}$. Red blood cells may be labelled by both of these methods for use in the determination of the circulating blood volume.

*Method.* The first of the 2 groups of animals used consisted of 12 adult Slonaker rats, of which 3 were lactating, the second group of 30 adult rats (the first generation of a cross-breeding of highly inbred Slonaker and Curtis Dunning rats). In each group the blood type of all animals was the same. The individuals of Group I were given doubly labelled red blood cells, those of Group II, cells labelled only with $P^{32}$.

A single animal was injected intraperitoneally with a solution of $Fe^{59}$, as ferric chloride, buffered with sodium citrate and adjusted to pH 6. After sufficient time had elapsed for the $Fe^{59}$ level in the blood to reach a constant value, approximately 5 cc was withdrawn into a heparinized syringe by cardiac puncture, placed in a paraffined 25 cc Erlenmeyer flask and incubated with $P^{32}$ as ($Na_2HP^{32}O_4$) at 37°C for 2 hours with constant rotation. The cells were washed 3 times by adding isotonic saline solution, centrifuging and removing the supernatant fluid. Then plasma containing no $P^{32}$ was added to the washed cells to reconstitute whole blood. Ether anesthetized animals in Group I and nembutal anesthetized animals in Group II were injected into the tail vein with 0.2 cc of this reconstituted blood. After 3 minutes approximately 1.0 cc of blood was withdrawn by cardiac puncture, 0.1 cc being used for the determination of $P^{32}$ content, 0.5 cc for the determination of the $Fe^{59}$ and 0.25 cc for the measurement of the hematocrit in Smith hematocrit tubes. Standards were prepared by diluting 0.2 cc of the reconstituted blood in 25 cc of distilled water.

The $P^{32}$ was assayed by drying 0.1 cc of blood on a piece of lens paper on a thin aluminum foil, wrapping the lens paper and aluminum foil in cellophane and counting on an Eck-Krebs counter-tube. In a similar manner, 0.1 cc of the diluted standard was mounted and counted. The probable error of

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[†] National Cancer Institute Post Doctorate Research Fellow.

[‡] A.E.C. Post Doctorate Research Fellow.

[§] Institute of Experimental Biology, University of California.

[1] Griffith, J. Q., and Campbell, R., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 38.

[2] Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. P., *J. Biol. Chem.*, 1933, **99**, 457.

[3] Cartland, G. F., and Koch, F. C., *Am. J. Physiol.*, 1928, **85**, 540.

[4] Went, S., and Drinker, C. K., *Am. J. Physiol.*, 1929, **88**, 468.

[5] Metcalf, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **141**, 697.

[6] Beckwith, J. R., and Chanutin, A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 66.

[7] Hahn, P. F., Bale, W. F., Hettig, R. A., and Whipple, G. H., *Science*, 1940, **92**, 131.

[8] Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H., *J. Exp. Med.*, 1939, **69**, 731.

[9] Hevesy, G., and Zerahn, K., *Acta Physiol. Scand.*, 1942, **4**, 376.

[10] Hahn, L., and Hevesy, G., *Acta Physiol. Scand.*, 1940, **1**, 1.

TABLE I.
The Blood Volume of the Adult Rat as Determined with Red Blood Cells Doubly-labelled with $Fe^{59}$ and $P^{32}$.

| Rat No.* | Body wt in g | Hematocrit | Blood vol. ($Fe^{59}$) cc/100 g body wt | Blood vol. ($P^{32}$) cc/100 g body wt | Total red cell vol. ($P^{32}$) cc/100 g body wt |
|---|---|---|---|---|---|
| 1 | 205 | 45. | 5.06 | 5.26 | 2.36 |
| 2 | 213 | 45.5 | 4.69 | 5.58 | 2.54 |
| 3 | 185 | 44 | 4.97 | 5.13 | 2.26 |
| 4 | 198 | 43.5 | 5.81 | 5.95 | 2.58 |
| 5 | 190 | 49 | 3.63 | 3.94 | 1.93 |
| 6 | 180 | 53 | 4.28 | 4.10 | 2.17 |
| 7 | 185 | 48 | 4.11 | 4.32 | 2.07 |
| 8 | 174 | 50 | 4.36 | 4.31 | 2.16 |
| 9 | 170 | 50 | 4.41 | 4.12 | 2.06 |
| 10 | 233 | 46 | 4.98 | 5.36 | 2.46 |
| 11 | 202 | 50 | 4.95 | 5.10 | 2.55 |
| 12 | 218 | 48 | 5.74 | 5.27 | 2.53 |
| Avg | 196.3 | 47.7 | 4.75 | 4.87 | 2.31 |

* Rats 1-9 were males; 10-12 lactating females.

counting was 1%. The $Fe^{59}$ was assayed by a modification of Peacock's method.[11] The blood samples and the diluted standard were placed in 25 cc Erlenmeyer flasks; 10 mg of carrier iron, as ferric chloride, were added to the sample which was then dried at 70°C. After drying, 3-5 cc of concentrated nitric acid were added and evaporated to dryness. The ashing was completed with concentrated sulfuric acid and 30% hydrogen peroxide. The sulfuric acid solution of the ash was transferred to a centrifuge cone and the iron precipitated with concentrated sodium hydroxide. After centrifuging, the supernatant was discarded, the precipitate was redissolved in 0.25 cc of concentrated hydrochloric acid, the precipitation and centrifugation repeated, and the supernatant again discarded. The final precipitate was dissolved in 0.25 cc concentrated hydrochloric acid. The solution was transferred to an electrodeposition cell containing 35 cc of plating solution (one part saturated oxalic acid and five parts saturated ammonium oxalate). The iron was electro-deposited at 0.8 amperes and 12 volts in an apparatus designed by Dunn.[12] The samples and the standard were counted on a thin end window Geiger-Mueller counter. The probable error of counting was 1%.

*Results.* The blood volume as determined by both $Fe^{59}$ and $P^{32}$, together with the total red cell volume, the hematocrit and the body weight of the Group I animals are presented in Table I. The same determinations for the Group II animals, except the $Fe^{59}$ volumes, are recorded in Table II. The coefficient of correlation between the blood volume and hematocrit was 0.64. The standard deviation for all of the animals was not significantly different from the standard deviation for groups of litter mates. The average blood volume as determined by $P^{32}$ for both groups, excluding the lactating females, was 4.59 ± 0.57 cc[‖]/100 g body weight; the total red cell mass 2.16 ± 0.20 cc[‖]/100 g body weight and the hematocrit 45.8 ± 2.9.[‖]

*Discussion.* The values reported using vital red are 4.3 cc/100 g body weight (1937)[1]; 6.38 cc/100 g body weight (1933)[2]; 6.9 cc/100 g body weight (1928)[3]; and 7.4 cc/100 g body weight (1929)[4]; with the use of Evans blue (T-1824)-7.6 cc/100 g body weight (1944)[5] and 7.98/100 g body weight (1941).[6] The total red cell volume was reported as 2.95 cc/100 g body weight;[2] 3.45 cc/100 g body weight;[5] and 3.90 cc/100 g body weight.[6]

The good agreement between the values of

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[11] Peacock, W. C., Evans, R. W., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J., II, *J. Clin. Invest.*, 1946, **25**, 605.

[12] Dunn, R., Donner Laboratory, Univ. of Calif.

[‖] Standard deviation.

TABLE II.
Blood Volume of the Adult Rat as Determined by $P^{32}$ Labelled Red Blood Cells.

| Rat No.* | Body wt in g | Hematocrit | Blood vol. ($P^{32}$) cc/100 g body wt | Total red cell vol. ($P^{32}$) cc/100 g body wt |
|---|---|---|---|---|
| 13 | 260 | 49 | 4.27 | 2.08 |
| 14 | 240 | 45 | 4.92 | 2.20 |
| 16 | 180 | 46 | 4.77 | 2.22 |
| 17 | 165 | 43 | 5.02 | 2.18 |
| 18 | 150 | 45 | 4.17 | 1.88 |
| 19 | 230 | 46 | 4.87 | 2.26 |
| 20 | 240 | 47 | 4.34 | 2.04 |
| 21 | 230 | 54 | 3.96 | 2.13 |
| 22 | 260 | 47 | 3.92 | 1.85 |
| 23 | 230 | 48 | 4.13 | 1.98 |
| 24 | 185 | 50 | 3.46 | 1.78 |
| 25 | 225 | 45.5 | 5.50 | 2.31 |
| 26 | 232 | 47 | 5.39 | 2.54 |
| 27 | 237 | 47 | 4.33 | 2.10 |
| 28 | 190 | 45 | 4.84 | 2.30 |
| 29 | 257 | 44 | 4.82 | 2.17 |
| 30 | 225 | 47 | 5.11 | 2.08 |
| 31 | 210 | 44 | 4.24 | 2.04 |
| 32 | 205 | 50 | 4.14 | 2.12 |
| 33 | 215 | 50 | 4.41 | 2.51 |
| 34 | 235 | 50 | 4.94 | 2.26 |
| 35 | 230 | 44 | 4.52 | 1.99 |
| 36 | 295 | 43 | 4.00 | 1.72 |
| 37 | 315 | 46 | 4.23 | 1.95 |
| 38 | 320 | 48 | 4.66 | 2.24 |
| 39 | 240 | 46 | 4.50 | 2.07 |
| 40 | 200 | 44 | 4.35 | 1.91 |
| 41 | 235 | 43 | 4.81 | 2.07 |
| 42 | 245 | 48 | 4.65 | 2.23 |
| 43 | 260 | 45 | 4.99 | 2.25 |
| 44 | 240 |  | 3.79 |  |
| Avg | 231.7 | 45.0 | 4.52 | 2.12 |

* Rats 13-14, 16-17, 19-23, 25-26, 27-30, 31-32, 33-34, 35-37, 39-40, 40-42, 24 and 44 were litter mates; 16, 17, 18, 24 and 44 were females.

the blood volume as obtained by $Fe^{59}$ and by $P^{32}$ labelled red blood cells permits the use of the $P^{32}$ labelled red blood cells for the determination of the blood volume of the rat. The use of $P^{32}$ thus eliminates the comparatively lengthy and tedious processes required for the accurate assay of $Fe^{59}$. In addition it eliminates the need for building up in a donor animal red blood cells with a concentration of $Fe^{59}$ sufficient so that a large dilution of a small amount of the donor cells may be accurately determined by measurement of the amount of $Fe^{59}$ present in the diluted sample.

*Conclusions.* 1. The blood volume of normal rats determined by the use of $Fe^{59}$ labelled red blood cells is essentially the same as with $P^{32}$ labelled cells.

2. The use of $P^{32}$ labelled red blood cells is hence satisfactory for the determination of blood volume.

3. Determinations with $P^{32}$ indicate that the blood volume of the normal rat is 4.59 ± 0.57/cc 100 g body weight; the total red cell volume 2.16 ± 0.20 cc/100 g body weight; and the hematocrit 45.8 ± 2.6.

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## 17124. Serum Cholinesterase in Hyperthyroidism.

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Conflicting reports have appeared in the literature concerning serum cholinesterase levels in hyperthyroidism. Antopol *et al.*,[1] reported an increased serum cholinesterase level in twenty-two cases of untreated hyperthyroidism and a return toward normal values in 13 cases treated by iodine or operation. Milhorat,[2] Butt[3] and Faber[4] reported normal values in several cases of hyperthyroidism. During preparation of this manuscript an abstract of a paper by Ambrus *et al.*[5] came to our attention. They recorded significant changes in the serum cholinesterase in diseases of the thyroid gland as observed clinically and experimentally. They found that thiourea derivatives lowered the high values in clinical and experimental hyperthyroidism.

It is the purpose of this communication to present our findings in normal and hyperthyroid patients.

*Methods.* The serum cholinesterase was determined in cases of euthyroidism, hyperthyroidism, and non-toxic adenoma. The diagnosis was made by clinical appraisal, laboratory findings, response to 6N-propylthiouracil and/or microscopic examination of the surgically excised thyroid glands.

Venous blood was allowed to clot, the serum separated and a few drops of chloroform added to the latter for preservation. The method of determining serum cholinesterase was a modification of Stedman's procedure[6] where the serum and substrate were titrated at a temperature between 36-37°C using m-cresol purple as the indicator. The titration was discontinuous, and was carried out at two 10 minute intervals. The average value obtained was used as the final figure.

*Results.* The range based on determinations in 33 normal adults, was 0.30-0.52 unit with the exception of 3 values (3 cases), 0.62, 0.62, and 0.22 unit. Of this series 23 were males in whom the range was 0.22 - 0.52 unit with an average of 0.42 unit. In the 10 females the comparative values were 0.39-0.62 with an average of 0.51 unit.

In Fig. 1 a summary of the findings is presented. Of the 18 cases of hyperthyroidism that were investigated 11 came under our observation before therapy was instituted or during therapy insufficient to produce an adequate clinical response. In this group the values ranged from 0.68 to 1.45 units and all but one case was higher than 0.70 unit. In 4 of these patients only an initial cholinesterase value was obtained. The remaining seven cases in this group were followed by subse-

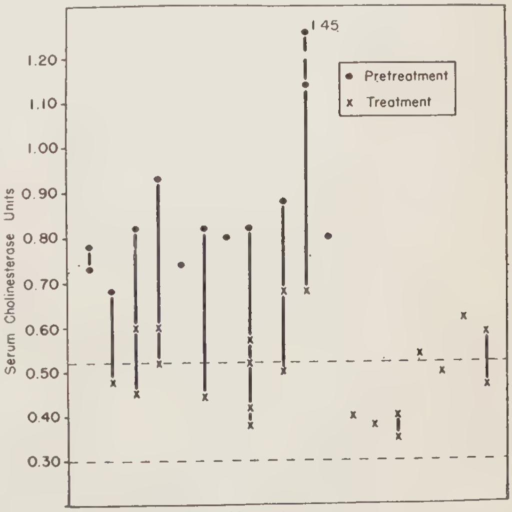

FIG. 1.

Summary of findings in 18 cases of hyperthyroidism, showing changes in serum cholinesterase with treatment.

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[1] Antopol, W., Tuchman, L., and Schifrin, A., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 46.

[2] Milhorat, A. T., *J. Clin. Invest.*, 1938, **17**, 649.

[3] Butt, H. R., Comfort, M. W., Dry, T. J., and Osterberg, A. E., *J. Lab. and Clin. Med.*, 1942, **27**, 649.

[4] Faber, M., *Acta Med. Scand.*, 1943, **114**, 59.

[5] Ambrus, K., and Ambrus, G., *Orvosok Lapja*, 1947, **3**, 865 (cited in *Excerpta Medica*, 1947, **1**, Abst. No. 473, p. 169).

[6] Stedman, E., Stedman, E., and Easson, L. H., *Biochem. J.*, 1932, **26**, 2056.

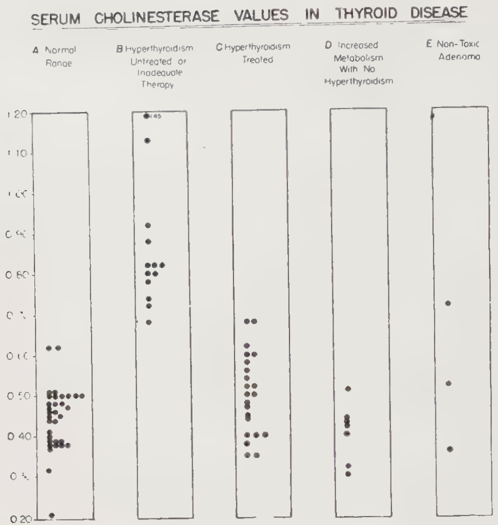

Fig. 2.

quent cholinesterase levels during adequate therapy. There appeared to be no correlation between the severity of the hyperthyroidism and the level of serum cholinesterase.

In 7 additional cases determinations were made only after adequate therapy had been instituted, so that a total of 14 cases was observed after good clinical response to antithyroid treatment. The values of this group of 14 cases were uniformly below 0.70 unit and varied from 0.35 to 0.68 unit. There were 3 types of cases in this category, 1) iodine and thyroidectomy (3 cases), 2) 6N-propyl-thiouracil, iodine and thyroidectomy (6 cases), 3) 6N-propyl-thiouracil alone (5 cases). All cases regardless of the type of therapy reacted in the same manner. In Fig. 2 is presented a summary of the results obtained in all cases.

Three cases of non-toxic adenoma were observed. In 2, normal values were obtained; a definitely high value was found in the third case. This finding is at present unexplained.

Of interest were the following 3 cases with increased basal metabolic rate in whom a clinical diagnosis of hyperthyroidism was initially entertained but subsequently ruled out.

1. Female, age 55; severe psychoneurosis, BMR plus 67%

2. Female, age 67: metastatic carcinoma of cervical lymphnodes with tracheal pressure symptoms; BMR plus 63, 66%.

3. Female, age 49; dyspnea, palpitation, fibrillation and pedal edema; BMR plus 52%, plus 63%; no response to 6N-propyl-thiouracil. Normal values for serum cholinesterase were found in each case.

No relationship was found between the serum cholinesterase level and BMR, cephalin cholesterol flocculation test, total serum protein or albumin and globulin. In this respect, our findings conflict with those of Faber,[7] who found a direct relationship between the cholinesterase activity and serum albumin. A tendency to an inverse relationship existed between the levels of the enzyme and serum total cholesterol.

*Discussion*. The data presented demonstrates an increase in the cholinesterase of the serum of hyperthyroid patients. Its significance is at present uncertain. In those cases in which 6N-propyl-thiouracil was used preoperatively, the serum cholinesterase returned to normal values concomitant with the improvement in clinical state and subsequent thyroidectomy did not cause a further decrease.

*Summary*. Observations on 18 cases of hyperthyroidism are presented. This condition is characterized by significantly increased levels of serum cholinesterase. These values returned toward the normal range with adequate antithyroid therapy. Of 3 patients with nontoxic adenoma of the thyroid, 2 were in the normal range and one showed a high value. No definite relationship was found between serum cholinesterase and basal metabolic rate, total serum protein, albumin, globulin or cephalin-cholesterol flocculation test. Due to the comparatively small series no conclusion can be drawn concerning sex differences in serum cholinesterase.

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[7] Faber, M., *Acta Med. Scand.*, 1943, **114**, 72.

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## 17125. The Xanthine Oxidase Activity of Rat Tissues.*

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The scattered distribution of xanthine oxidase in various organs of different species has been summarized by Morgan.[1] In general, it is absent from embryonic tissue, but appears shortly after birth. It is present in the livers of most, but not all, species and is encountered frequently in kidney, spleen, lung, small intestine and occasionally in pancreas. It has not been detected in muscle, thymus, stomach or large intestine of those species studied.

The purpose of the present communication is to consider 1) the relative amounts of xanthine oxidase in various rat tissues, and 2) the changes in the enzyme activity of these tissues as the liver xanthine oxidase is depleted by dietary means.

*Methods.* Adult rats weighing 200-300 g were used throughout. Normal xanthine oxidase levels were obtained in rats maintained on Purina dog chow. Liver xanthine oxidase was depleted by feeding rats a purified 8% casein diet[2] for 4 to 8 weeks. The xanthine oxidase activity of various organs was determined by the method of Axelrod and Elvehjem.[3] Added xanthine oxidase was prepared from milk by the method of Ball.[4]

*Results.* Table I shows the average xanthine oxidase activity of the normal rat tissues studied, together with the effect of adding methylene blue or purified xanthine oxidase. The amount of enzyme present in each tissue giving rise to the activity actually found could not be evaluated by a direct comparison of activities. Added xanthine oxidase was recovered nearly quantitatively when added to lung, spleen, or kidney, but was recovered in increased amounts when added to liver; hence a given weight of enzyme was more active in liver than in the other tissues. The enzyme was destroyed in the presence of homogenized small intestine; not only was the added xanthine oxidase not recovered, but in many determinations the activity fell off rapidly. All values for small intestine were therefore minimal, but alterations with diet were comparable because they were all determined in the same way.

The effect of adding 0.15 cc of 0.0113 M methylene blue in the aerobic determination of xanthine oxidase was not the same for all tissues. When added to the isolated milk xanthine oxidase or to liver homogenate, methylene blue increased the activity by an average of 3.0 and 1.6 times respectively.

TABLE I.
Xanthine Oxidase Activity of Normal Rat Tissues.

| | No. of determinations | Xanthine oxidase activity | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CmmO$_2$/g dry wt/hr | | | CmmO$_2$/20 min. | | | |
| | | Range | Mean ± S.E. | M. blue added | Present in tissue | X.O. added | Total found | X.O. recovered |
| Lung | 7 | 395–630 | 479 ± 30 | 500 | 12 | 17 | 27 | 15 |
| Spleen | 8 | 390–665 | 534 ± 35 | 525 | 13.5 | 17 | 29 | 15.5 |
| Kidney | 7 | 0–195 | 135 ± 23 | 345 | 3.5 | 17 | 18.5 | 15 |
| Small intestine | 8 | 450–810 | 628 ± 42 | 1150 | 14 | 17 | 17 | 3 |
| Stomach | 6 | 0–85 | 42 ± 19 | 120 | — | — | — | — |
| Liver | 12 | 1475–2320 | 1862 ± 75 | 2790 | 53 | 18.5 | 83 | 30 |

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* Aided by a grant from the Nutrition Foundation, Inc.

[1] Morgan, E. J., *Biochem. J.*, 1926, **20**, 1282.

[2] Westerfeld, W. W., and Richert, Dan A., *Science*, 1949, **109**, 68.

[3] Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **140**, 725.

[4] Ball, E. G., *J. Biol. Chem.*, 1939, **128**, 51.

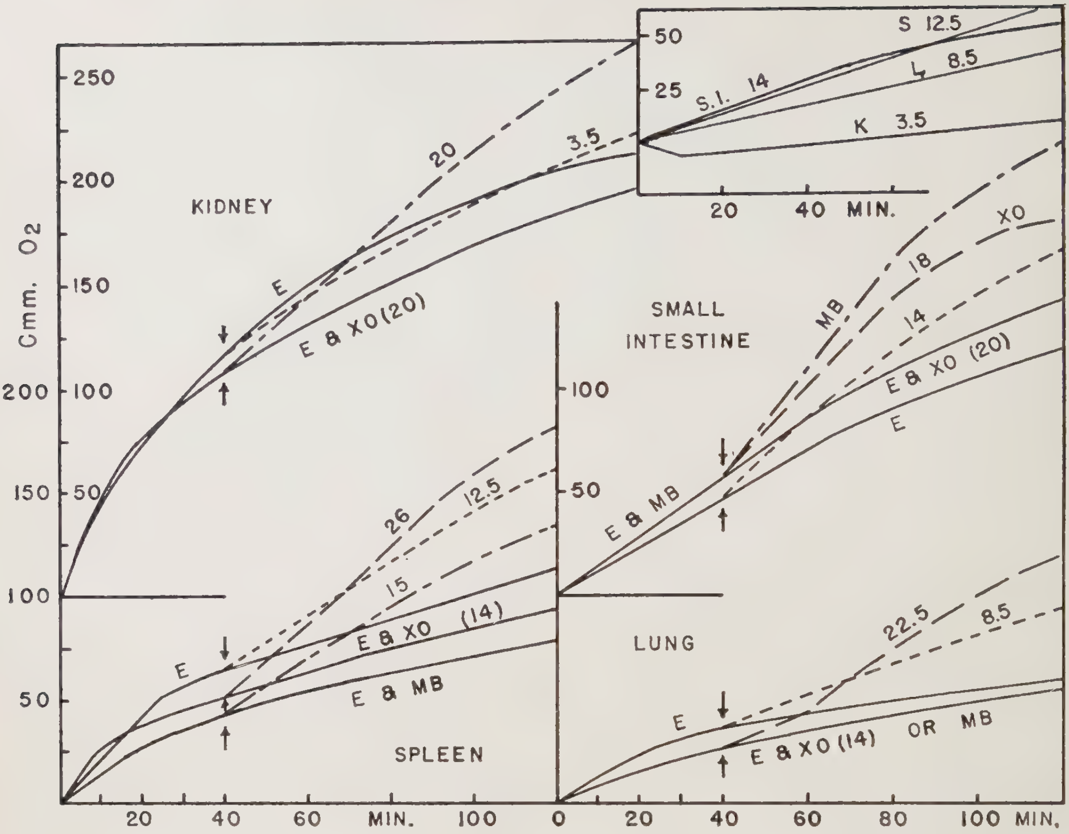

FIG. 1.

Typical oxygen consumption curves for kidney, spleen, small intestine and lung during the determination of xanthine oxidase activity. The effect of adding purified xanthine oxidase or methylene blue to the main body of the Warburg flask is also illustrated.

Solid lines = Endogenous respiration (E) with or without added xanthine oxidase (X.O.) or methylene blue (MB).

Dotted lines = Respiration after tipping in xanthine at the arrows.

Numbers along curves refer to net oxygen uptake in cmm per 20 minutes due to oxidation of xanthine.

Box, upper right: Net oxygen consumption curves due to oxidation of xanthine.

Kidney, small intestine and stomach also gave appreciable increases in xanthine oxidase activity in the presence of methylene blue, and this was very useful in demonstrating the presence of this enzyme in kidney and stomach where the amount was often so small as to be questionable. The xanthine oxidase activity of lung and spleen were unchanged in the presence of methylene blue. The significance of the differences between tissues in respect to this methylene blue effect is not yet apparent.

Brain, skeletal muscle and testes were devoid of xanthine oxidase activity by the usual test as well as in the presence of methylene blue aerobically. Skin contained 100 to 200 units of xanthine oxidase activity (Cmm$O_2$/g/hr), and methylene blue increased the activity. Skin xanthine oxidase was not studied in detail because of the difficulty in homogenizing this tissue.

Fig. 1 shows typical oxygen consumption curves for kidney, spleen, lung and small intestine during the determination of xanthine oxidase activity. The effect of adding purified xanthine oxidase or methylene blue to the main body of the Warburg flask is also illustrated. Similar studies with liver will be reported in another communication.

Fig. 2 shows the effect of a purified low-protein diet on the xanthine oxidase activity

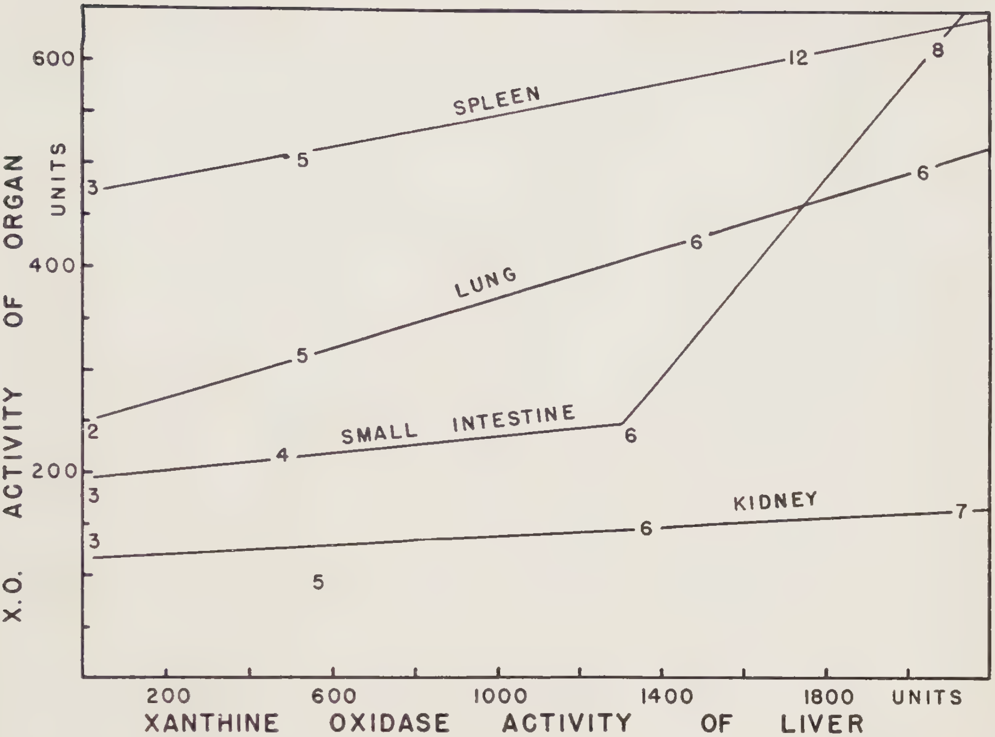

Fig. 2.

Changes in the xanthine oxidase activity of spleen, lung, small intestine and kidney as compared with the changes produced in liver xanthine oxidase by feeding a purified 8% casein diet. Numbers along curves refer to the number of determinations that were averaged to obtain each point on the curve.

of these various tissues in comparison with the changes observed in the liver.

Individual rats varied greatly in length of time on the diet required to deplete the liver of xanthine oxidase. At 4 to 5 weeks, 11 rats had 0, 0, 0, 0, 385, 395, 455, 635, 705, 1160, and 1580 units of liver xanthine oxidase; after 8 weeks, 4 of 5 rats had no liver xanthine oxidase activity. Inanition, which is known to decrease the liver xanthine oxidase,[5] was not a factor in these experiments with adult rats, since body weights increased consistently on the purified low protein diet. Rats were analyzed arbitrarily after 2 to 8 weeks on the diet, and the results were then correlated on the basis of the liver xanthine oxidase found; the data from rats with similar liver xanthine oxidase activities were grouped together without regard to the duration of the individual dietary periods.

As the liver xanthine oxidase activity fell from over 2000 units to zero the small intestine lost about 2/3 of its activity rapidly, but then remained fairly constant at the lower level; lung lost about ½ of its activity in a gradual manner; losses in the spleen and kidney were 20-30%, which were not enough to be statistically different from the normal levels in this limited series. That portion of the enzyme which could be lost readily from the small intestine was depleted more rapidly than the liver. The xanthine oxidase activities of the other tissues were much more refractory to dietary changes than was the liver enzyme.

Assuming that the intact structures maintain the same relative enzyme activities as exhibited in the homogenates, then the total xanthine oxidase activity of a normal mature rat would be distributed approximately as follows: 67% in liver, 20% in skin, 8%

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[5] Miller, L. L., *J. Biol. Chem.*, 1948, **172**, 113.

in small intestine, 2% each in spleen and lung, and 1% in kidney. When the liver has been depleted to zero xanthine oxidase activity by a purified low protein diet, the entire rat has lost about 75% of its original enzyme activity (assuming the skin is relatively unchanged).

*Summary.* The average xanthine oxidase activities in CmmO$_2$/g dry weight/hour for adult rat tissues were: Liver 1862, small intestine 628, spleen 534, lung 479, kidney 135, stomach 42, skin 100-200, brain 0, muscle 0, testes 0. Two-thirds of the total activity in the entire rat was in the liver.

Methylene blue added to the Warburg flask in the aerobic determination increased the xanthine oxidase activity in liver, kidney, stomach and small intestine, but not in lung or spleen. Added xanthine oxidase was recovered quantitatively in the presence of lung, spleen and kidney; greater than added amounts of activity were recovered from liver and decreased amounts from small intestine.

As the liver xanthine oxidase was depleted by a purified low protein diet, the small intestine lost about 2/3 of its activity, and lung lost about ½. Losses from the spleen and kidney were small. The entire rat lost ¾ or more of its xanthine oxidase activity.

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## 17126. Relationship Between Protein Osmotic Pressure and Density in Plasma from Cats, Dogs, and Humans.

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*From the Department of Physiology, Harvard Medical School.*

During the course of experiments on the effective osmotic pressure of the plasma proteins in the capillary circulation[1] we have had occasion to compare protein osmotic pressure with density in a large number of samples of plasma from cats, dogs, and humans. We were surprised to find that for any given density the protein osmotic pressure of human plasma is higher than that of plasma from cats or dogs. The data given below may prove useful to other investigators who wish to use plasma density as a measure of the osmotic activity of the plasma proteins in these species.

*Methods.* Blood was drawn from the carotid arteries of cats and dogs anesthetized with Nembutal. Human blood was obtained by venipuncture. Heparin was used to prevent coagulation. Low protein concentrations were obtained by diluting the plasma with Ringer's solution (0.9% NaCl, 0.042% KCl, 0.024% CaCl$_2$, 0.020% NaHCO$_3$) of density 1.0068 relative to water at the same temperature. High protein concentrations were obtained by evaporating the plasma in cellophane bags placed in front of a fan. The bags were then sealed close to the liquid level and the concentrated plasma dialyzed against cold Ringer's solution until ionic equilibrium was established as indicated by the electrical conductivity.

Densities were determined by the Falling Drop Method of Barbour and Hamilton.[2] The standard deviation from the means of 68 duplicate determinations performed with this method was $\pm 0.00013$ g/cc. Protein osmotic pressure measurements were made across collodion membranes using a Hepp osmometer.[3] All the results were corrected to 37°C by multiplying the observed values by the factor $273 + 37 \div 373 + t$, in which $t =$ room

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* Fellow of the Rockefeller Foundation (1946-48). Present address: Department of Physiology, University of Caracas, Venezuela.

[1] Pappenheimer, J. R., and Soto-Rivera, A., *Am. J. Physiol.*, 1948, **152**, 471.

[2] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

[3] Hepp, O., *Z. f. d. Gesamte Exp. Med.*, 1936, **99**, 709.

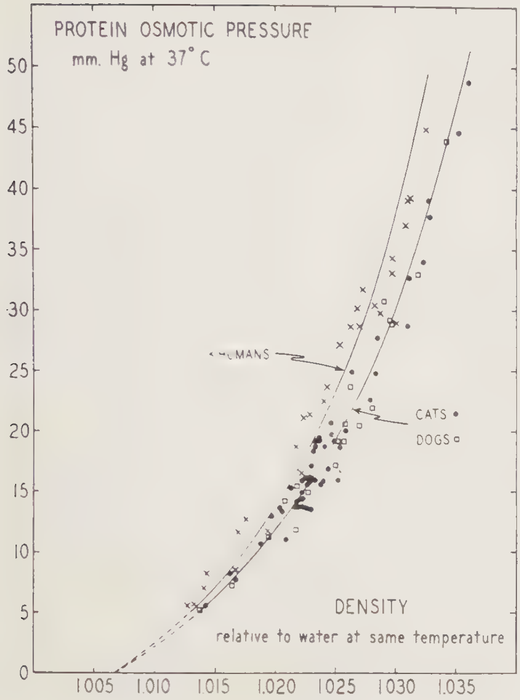

Fig. 1.

temperature °C. The standard deviation from the means of 68 duplicate measurements by this method was ± 0.4 mm Hg. The pH of the plasma samples ranged from 7.2 - 7.5 (glass electrode). For purposes of calculation, however, a value of 7.4 was assumed since a variation of ± 0.3 pH unit produces a change of protein osmotic pressure too small to be detected with our apparatus (cf. also Equation 1)

*Results.* Fig. 1 shows the relations between density and protein osmotic pressure in the three species. It is seen that at any given density human plasma has a higher protein osmotic pressure than that of cat or dog plasma. There is no significant difference in this respect between cat plasma and dog plasma.

*Discussion.* The following analysis of the data leads to useful formulae relating protein osmotic pressure to density and provides a basis for interpretation of the results.

As shown by Scatchard *et al.* [4,5] protein osmotic pressure is related to protein concentration by an equation of the form:

$$\pi = \frac{\pi_o\ (1 - .649)\ C}{1 - (.004 + .009\text{pH})C} \qquad (1)$$

where $\pi$ = protein osmotic pressure mm Hg, $\pi_o$ = protein osmotic pressure per gram protein at infinite dilution, g = ratio of globulin to total protein and C = total protein concentration in g/100 cc. For human plasma at pH 7.4 the constants are such that

$$\pi = \frac{1.97C}{1 - 0.071C} \qquad \text{............}(2)$$

in which C is assumed to be 6.25 times the protein nitrogen. Protein concentration is related to density by an equation of the form:

$$C = K\ (\rho - \rho_o) \qquad \text{..........}\ (3)$$

in which $\rho_o$ is the density at zero protein concentration. In the present experiments in which plasma was diluted with Ringer's solution $\rho_o = 1.0068$

Substituting Equation 3 into Equation 2 we have

$$\pi = \frac{1.97\ K\ (\rho - 1.0068)}{1 - 0.071K\ (\rho - 1.0068)} \qquad \text{....}(4)$$

The curve conforming to Equation 4 which best fits our experimental data for human plasma (method of least squares) is drawn in Fig. 1. It leads to a value for K = 353 which may be compared with values ranging from 343-360 found by previous investigators who compared protein concentration (actually protein nitrogen) with density.[6-9] For the practical purpose of predicting protein osmo-

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[4] Scatchard, G., Batchelder, A. G., and Brown, A., *J. Clin. Invest.*, 1944, **23**, 459.

[5] Scatchard, G., private communication.

[6] Moore, N. S., and VanSlyke, D. D., *J. Clin. Invest.*, 1930, **8**, 337.

[7] Phillips, R. A., VanSlyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M., Copper sulfate method for measuring specific gravities of whole blood and plasma, Josiah Macy, Jr. Foundation, New York, 1945.

[8] Lowry, O. H., and Hunter, T. H., *J. Biol. Chem.*, 1945, **159**, 465.

[9] Kagan, B. M., *J. Clin. Invest.*, 1938, **17**, 373.

tic pressure from density in human plasma Equation 4 becomes

$$\pi = \frac{695\ (\rho - 1.0068)}{1 - 25\ (\rho - 1.0068)} \pm 2.4 \text{ mm Hg} \quad (5)$$

An examination of the partial specific volumes of the purified protein components of plasma by Oncley, Scatchard and Brown[10] has shown that the average specific volume of the proteins is not less than 0.74 cc/g. Since K is related to specific volume ($V\sigma$) by the relation

$$K = \frac{1}{1 - \rho_0\ V\sigma} \quad \text{........}(6)$$

we may predict that the minimum value of K for whole plasma is 389 and not 350 ± 10, as found in this and previous investigations cited above. An explanation of this discrepancy may be found in the recent studies of Armstrong, Budka, Morrison and Hasson[11] who report that protein in plasma is associated with (nitrogen-free) lipids so that the factor relating protein concentration to protein nitrogen in human plasma is closer to 6.8 than 6.25. Correction of K by the factor 6.8/6.25 yields a value of $K = 6.8/6.25 \times 353 = 385$ which is in close agreement with the value 389 predicted from the partial specific volumes of the individual components.

A similar analysis for dog and cat plasma cannot at present be made because the constants essential to Equation 2 have not been evaluated and because the effects of lipids on the factor relating protein nitrogen to protein concentration has not been determined for these species. However, for the practical purpose of calculating the osmotic pressure from density it is reasonable to suppose that the equation is of the same form as Equation 5. The curve defined by an equation of this form which best fits our experimental data for cat and dog plasma is drawn in Fig. 1. For these species

$$\pi = \frac{625\ (\rho - 1.0068)}{1 - 22.5\ (\rho - 1.0068)} \pm 1.7 \text{ mm Hg} \quad \text{....}(7)$$

The observed differences between human plasma and cat and dog plasma shown in Fig. 1 and in Equations 5 and 7 are in the direction to be expected if cat and dog plasma contain more lipid associated with plasma protein than does human plasma. Until further data are available, however, it is not possible to state that this is the sole factor involved.

*Summary.* 1. Data are given showing the relationship between protein osmotic pressure and density in plasma from normal humans, cats, and dogs. Formulae are derived which allow the prediction of protein osmotic pressure from density in each of the three species (Equations 5 and 7). 2. For any given density human plasma has a higher protein osmotic pressure than does plasma from cats or dogs. Possible reasons for this species difference are discussed.

I wish to express my gratitude to Dr. J. R. Pappenheimer for his invaluable help and suggestions throughout this work.

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[10] Oncley, J. L., Scatchard, G., and Browne, A., *J. Phys. and Colloid Chem.*, 1947, **51**, 184.

[11] Armstrong, S. H., Budka, M. J. E., Morrison, K. C., and Hasson, M., *J. Am. Chem. Soc.*, 1947, **69**, 1747.

## 17127. Xanthine and Mercurial Diuretics and Renal Tubular Transport of Glucose and P-aminohippurate in the Dog.*

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Mercurial and probably xanthine diuretics[1] inhibit certain enzyme systems in the kidney. It is probable that the diuretic action of these agents is due to their selective accumulation in the kidney in concentrations sufficient to inhibit the systems concerned with the reabsorption of water and sodium chloride. The action of these compounds on other renal transport mechanisms has not received much attention. It has been reported[2,3] that mersalyl (Salyrgan) depresses the maximal rate of transfer of diodrast and p-aminohippurate

TABLE I.
Effects of Salyrgan and Mercuhydrin on Transfer Maxima of Glucose and PAH.

| Dog No. | Wt (kg) | Date | Control GF* cc/min. | Control TmG* mg/min. | Control TmPAH* mg/min. | During diuresis GF* cc/min. | During diuresis TmG* mg/min. | During diuresis TmPAH* mg/min. |
|---|---|---|---|---|---|---|---|---|
| Tm-1 | 17 | 7– 2–48 | 53 | 212 | | 62 | 228 | |
| 1 | 17 | 11–23– | 65 | 245 | | 79 | 254 | |
| 1 | 17 | 12–17– | 54 | | 11 | 61 | | 14 |
| 1 | 17 | 1–13–49 | 61 | | 10 | 70 | | 13 |
| 2 | 17 | –20– | 63 | | 14 | 68 | | 15 |
| 3 | 15 | 10–22–48 | 65 | 215 | | 63 | 198 | |
| 3 | 15 | 11– 1– | 52 | | 9 | 44 | | 10 |
| 3 | 15 | 12– 3– | 56 | 196 | | 64 | 220 | |
| 3 | 15 | –29– | 53 | | 10 | 63 | | 10 |
| 4 | 15 | 11–18– | 67 | 232 | | 61 | 220 | |
| 6 | 14 | 7–16– | 47 | 233 | | 42 | 228 | |
| 6 | 14 | –22– | 42 | | 10 | 45 | | 9 |
| 7 | 16 | – 9– | 60 | 225 | | 47 | 205 | |
| 7 | 16 | 8–18– | 55 | 200 | | 48 | 172 | |
| 7–F | 15 | 4–29– | 52 | 193 | | 49 | 180 | |

* Average of three 10-minute periods.

TABLE II.
Effects of Theophylline on Transfer Maxima of Glucose and PAH.

| Dog No. | Wt (kg) | Date | Control GF* cc/min. | Control TmG* mg/min. | Control TmPAH* mg/min. | During diuresis GF* cc/min. | During diuresis TmG* mg/min. | During diuresis TmPAH* mg/min. |
|---|---|---|---|---|---|---|---|---|
| Tm-1 | 15 | 7– 2–48 | 66 | | 10 | 58 | | 11 |
| 1 | 15 | – 7– | 53 | 212 | | 60 | 230 | |
| 1 | 15 | 12–17– | 50 | | 11 | 57 | | 14 |
| 3 | 15 | 9– 9– | 53 | | 11 | 57 | | 12 |
| 3 | 15 | –16– | 51 | 187 | | 47 | 173 | |
| 6 | 14 | 7–16– | 47 | 232 | | 42 | 225 | |
| 6 | 14 | –23– | 52 | | 11 | 58 | | 10 |
| 7 | 17 | 6–23– | 71 | 261 | | 64 | 242 | |
| 6-F | 16 | 11–13– | 60 | 252 | | 62 | 270 | |

* Average of three 10-minute periods.

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

[1] Handley, C. A., unpublished data.

[2] Brun, C., Hilden, T., and Raaschow, F., *Acta Pharmacol. Toxicol.*, 1947, **3**, 1.

[3] Berliner, R. W., Kennedy, T. J., and Hilton, J. G., *Am. J. Physiol.*, 1948, **154**, 537.

(PAH) in man although the TmPAH in the dog is not influenced by mersalyl.[3] The following data concerns the measurement of glucose and PAH Tm's in normal dogs and during the period of maximal diuresis by mercurial diuretics and theophylline.

*Methods.* Trained, unanesthetized, female dogs weighing 14-17 kg were used. Glomerular filtration rate (GF) was measured by creatinine. Glucose and p-aminohippurate Tm's were measured in independent experiments because of the reported mutual interference in the tubular transfer of these substances.[4,5]

Creatinine was determined by the method of Folin and Wu,[6] p-aminohippurate by the method of Bratton and Marshall[7] and glucose by Hanes' modification of Hagedom and Jensen's method.[8] The dose of meralluride (Mercuhydrin) and mersalyl, the two mercurials used, was 0.1 cc per kg (4 mg per kg of combined mercury) and 10-20 mg per kg of theophylline was employed.

*Results.* The results recorded in Tables I and II show that neither theophylline nor the mercurial diuretics studied interfere with the renal tubular transfer mechanisms for glucose or p-aminohippurate. These results indicate that the energy yielding mechanism concerned with the reabsorption of water and sodium chloride in the dog is distinct from the mechanisms concerned in the transfer of glucose and PAH. During this investigation it was observed that it is possible to reduce the TmG and TmPAH indirectly by diuretics.[1] This effect is due to dehydration and may be produced by the saline diuretics as well as the mercurial and xanthine groups, if sufficient urine output is produced to cause dehydration.

*Summary.* Mercuhydrin, salyrgan and theophylline, in diuretic doses, do not interfere with the renal tubular transfer of glucose or p-aminohippurate in the dog.

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[4] Klopp, C., Young, N. F., and Taylor, H. D., *J. Clin. Invest.*, 1945, **24**, 117.

[5] Houck, C. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 398.

[6] Folin, O., and Wu, H. J., *J. Biol. Chem.*, 1919, **38**. 81.

[7] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

[8] Hanes, C. S., *Biochem. J.*, 1929, **23**, 99.

## 17128. Absorption of Manganese by the Thyroid Gland of the Guinea Pig.

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Ray and Deysach[1] found that subcutaneous injection of manganese chloride into guinea pigs resulted in the accumulation of manganese in varying amounts in all organs tested, but that the accumulation was especially great in the thyroid gland where deposition was in direct proportion to the dose administered. Injections of large doses of the salt depressed the oxygen consumption of the animal and small doses augmented it. Noticeable effects within a few minutes after administration suggested early absorption by the thyroid gland. The purposes of the present study were to find the exact time interval for the appearance of manganese in the thyroid following its subcutaneous injection, to determine the rate at which it is deposited in this organ during a 24 hour period, and finally to obtain information on its rate of elimination.

*Experimental.* Each of 27 guinea pigs was given one subcutaneous injection of 10.0 mg of manganese chloride per kg of body weight. The animals were sacrificed 3 at a time, at intervals ranging from 15 minutes to 4 days following the injection, and the manganese

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[1] Ray, T. W., and Deysach, L. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 228.

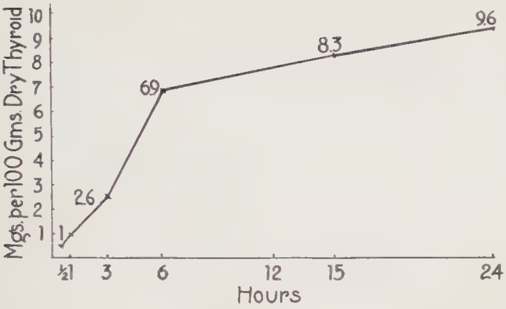

FIG. 1.

Manganese deposition in the thyroid gland of the guinea pig following a single subcutaneous injection of manganese chloride in a dosage of 10.0 mg per kg of body weight.

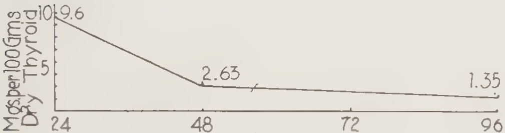

FIG. 2.

Rate of elimination of manganese from the thyroid gland of the guinea pig.

content of the thyroid gland was determined by the procedure of Ray.[2] The results are recorded graphically in Fig. 1 and 2. Each point on the graphs represents the average amount of manganese found in the thyroids of 3 animals. A detectable increase over the level found in earlier tests[1] was noted in 30 minutes, and a measurable increment was found in one hour. Fig. 1 shows further that the accumulation of manganese was rapid during the early stages of absorption. Of the total amount deposited in a 24 hour period, about 70% of it accumulated within the first 6 hours. Fig. 2 shows that the elimination of manganese from the thyroid, once it began, was very rapid. More than 70% of the 24 hour accumulation disappeared within 48 hours after the injection, and 85% of it disappeared within 96 hours.

*Summary.* Subcutaneous injection of manganese chloride into the guinea pig caused manganese to accumulate in all organs of the body tested, but the deposition was especially great in the thyroid gland where it began to appear within 30 minutes after administration. Absorption was particularly rapid during the first few hours, but the element continued to accumulate for at least 24 hours. Of the total amount of manganese deposited in the thyroid gland about 70% accumulated during the first 6 hours. Elimination of manganese from the thyroid, once it began, was rapid. Seventy per cent of the 24 hour accumulation disappeared within 48 hours after the injection; 85% of it vanished in 96 hours.

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[2] Ray, T. W., *J. Biol. Chem.*, 1940, **134**, 677.

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## 17129. Effect of the D- and L-isomers of Isoamidone* on the Permeability of Dog Erythrocytes.†

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We have previously reported that changes in the permeability or erythrocytes *in vitro* appeared to be correlated with changes in the activity of the acetylcholine-cholinesterase system in the red cell.[1,2] This conclusion was based on the observation that physostigmine as well as methadon,§ both of which are chol-

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* 1-dimethylamino-2-methyl-3,3-diphenyl hexanone-4.

† Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

‡ U. S. Public Health Fellow.

[1] Greig, M. E., and Holland, W. C., submitted for publication to *Arch. Biochem.*

[2] Greig, M. E., and Holland, W. C., *Fed. Proc.*, 1949, **8**, 297.

§ Methadon, amidone, 2-dimethylamino-4,4-diphenyl-heptanone 5. The methadon and isoamidone were kindly supplied by the Mallinckrodt Chemical Works.

TABLE I.

Effect of the D- and of the L-isomers of Isoamidone on Cholinesterase Activity of Dog Erythrocytes.

The media used were Ringer Krebs bicarbonate saline (R.K.),[7] sodium bicarbonate saline (Na) consisting of 100 parts 0.9% NaCl and 21 parts 1.3% $NaHCO_3$, and potassium bicarbonate saline (K) consisting of KCl and $KHCO_3$ in the same proportions as for the sodium buffer. 0.2 cc packed dog erythrocytes, and acetylcholine in a final concentration of 0.01 M in a volume of 2 cc were used. Carbon dioxide evolution was measured in Warburg manometers.

| Medium | Duration of exp., min. | Conc. of drug, M | | Mm$^3$ $CO_2$ evolved | | | | |
|---|---|---|---|---|---|---|---|---|
| | | D | L | Control | D | % effect | L | % effect |
| Na | 60 | .006 | .0002 | 115 | 84 | 27 | 84 | 26 |
| K | 60 | .006 | .0002 | 124 | 87 | 30 | 67 | 46 |
| RK | 60 | .00087 | .00087 | 202 | 124 | 39 | 98 | 51 |
| RK | 60 | .00087 | .00087 | 228 | 168 | 26 | 102 | 55 |

TABLE II.

Effect of the D- and the L-isomers of Isoamidone on Glycolysis.

The experiments were carried out in a Ringer Krebs bicarbonate medium[7] in Warburg manometers. The concentration of glucose in the experiment with erythrocytes was 0.014 M, that in the experiment with brain 0.01 M. The amount of tissue was 1.0 cc packed dog erythrocytes or 150 mg homogenized rat brain in a final volume of 2 cc. The gas phase was 95% $N_2$, 5% $CO_2$.

| Tissue | Duration of exp., min. | Conc. of isoamidone, M | $CO_2$ evolved in mm$^3$ | | | | |
|---|---|---|---|---|---|---|---|
| | | | Control | D | % effect | L | % effect |
| Erythrocytes | 40 | .00174 | 89 | 70 | 21 | 77 | 14 |
| Rat Brain | 60 | .00174 | 130 | 107 | 18 | 101 | 22 |

inesterase inhibitors[3,4] produced changes in permeability of dog erythrocytes under certain conditions. Physostigmine is considered to be a specific inhibitor of cholinesterase. This drug produced most marked changes in the permeability of erythrocytes under the conditions where it had its maximum effect on cholinesterase activity. Methadon is not a specific inhibitor of cholinesterase as it also inhibits glycolysis of glucose[5] as well as certain oxidative processes.[6] However, under our experimental conditions, the inhibition of cholinesterase rather than the inhibition of glycolysis by methadon seemed to account for its effect on permeability of erythrocytes.

As the D- and L-isomers of isoamidone have been found to be equally effective in inhibiting glycolysis, but the L-isomer is a much more effective inhibitor of cholinesterase than is the D, it was assumed that if the effects on permeability were different with the different isomers, this action could be more readily attributed to their effect on cholinesterase than to that on glycolysis.

The present communication describes experiments dealing with the effects of the D- and of the L-isoamidone on the permeability of dog erythrocytes.

*Results.* In Table I are presented results of experiments on the effects of the D- and L-isomers of isoamidone on the cholinesterase of dog erythrocytes. As in the case of brain cholinesterase the L-isomer has a much greater inhibitory action than has the D- in the same concentrations.[3]

From Table II it may be seen that there is little or no difference between the effects of the D- and L-isomers on glycolysis of glucose by rat brain or by dog erythrocytes.

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[3] Greig, M. E., and Howell, R. S., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 352.

[4] Eadie, G. S., Bernheim, F., and Fitzgerald, D. P., *J. Pharm. Exp. Therap.*, 1948, **94**, 19.

[5] Greig, M. E., *Arch. Biochem.*, 1948, **17**, 129.

[6] Greig, M. E., and Howell, R. S., *Arch. Biochem.*, 1948, **19**, 441.

[7] Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, 1932, **210**, 33.

In our experiments the hemoglobin liberated has been taken as a criterion for changes in permeability of erythrocytes. If percent hemolysis be plotted against time, six possible arrangements of curves representing the effects of the D- and L-isomers of isoamidone on erythrocytes could be obtained. If C represents the hemolysis in the control suspension, D- that with the D-isomer and L- that with the L-isomer the 6 possible types obtainable would be (1) $L>D>C$, (2) $D>L>C$, (3) $C>D>L$, (4) $C>L>D$, (5) $D>C>L$, (6) $L>C>D$.

Experimentally we found that all 6 types occurred to some extent under different conditions. The factors controlling the type of curve obtained appeared to be mainly the proportions of Na and K in the medium and the pH. There seemed to be some variation in results obtained with red cells from different dogs under the same experimental conditions, although the results obtained with different samples of blood from the same dog under the same experimental conditions were quite consistent.

In a medium containing a high proportion of KCl compared with NaCl, curves of type 3 were most frequently obtained, while those of type 5 and type 1 occurred less often. Of 23 experiments carried out in a medium consisting of isotonic KCl or a mixture of isotonic KCl and isotonic NaCl in the proportions of 9:1, fifteen fell in type 3 and 8 in type 5. It was frequently observed that early in an experiment the results would follow curves of type 3, later the curves would cross and follow those of type 5 and still later those of type 1 and occasionally type 2.

In a medium containing a high proportion of Na compared with K there was little difference in the effects produced by D- and L-isomers. Usually curves of types 1 and 2 were obtained.

Results of 3 typical experiments are presented graphically in Fig. 1-3.

*Discussion.* It may be seen from the above results that:

1. the D- and L-isomers of isoamidone in the same concentrations inhibit the glycolysis of glucose by rat brain and by dog erythrocytes to about the same extent.

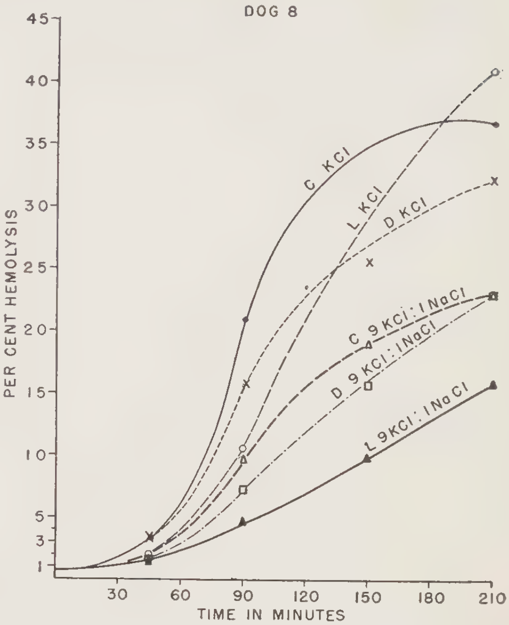

Fig. 1.

One cc packed dog erythrocytes were suspended in 10 cc isotonic saline in Erlenmeyer flasks. The final concentration of the isoamidone was 0.00022 M, that of acetylcholine 0.01 M. Samples of suspension of 1 cc were removed at intervals and after dilution and centrifugation the hemoglobin content of the supernatant fluid was determined using a photoelectric colorimeter.

C—Control.
D—D-isoamidone.
L—L-isoamidone.
Ac ch—Acetyl choline.

2. the D- and L-isomers of isoamidone inhibit the cholinesterase activity of erythrocytes to different extents, the L- being a more effective inhibitor than the D-isomer.

3. the D- and L-isomers of isoamidone have different effects on the permeability of dog erythrocytes.

As the stereoisomers have the same chemical properties, *e.g.*, molecular weight, solubility, molecular radius, rate of diffusion, the different effects on the permeability of erythrocytes could not be attributed to these factors.

If one assumes that the permeability of living cells is controlled by metabolic activity, then of the two metabolic reactions investigated which are affected by isoamidone the inhibition of cholinesterase activity would seem best able to account for the changes in permeability in view of the different effects produced by the two isomers on this enzyme.

In a medium containing a high proportion of KCl compared with NaCl the L-isomer usually produced greater resistance to hemolysis than the D at the start of the experiment, (type 3 and Fig. 1).

In a bicarbonate medium, pH 8, the L usually increased hemolysis, relative to the control and the D-isomer (Fig. 2). These results are similar to those found with physostigmine.[1,2] Occasionally curves of type 5 were found early in the experimental period, in which the L-isomer caused increased resistance and the D-increased fragility relative to the control.

The addition of acetylcholine increases the resistance of the control suspension in a K medium and tended to increase the differences between the effects produced by the D- and L-isomers relative to the control (Fig. 2).

In a medium consisting of either NaCl or high Na compared with K the differences between the effects of the D- and L-isomers were usually less marked (Fig. 3) and the D occasionally produced slightly greater hemolysis than the L (curves of type 2).

We also found that physostigmine had little effect on permeability in a medium of NaCl as evidenced by hemolysis but that it produced very marked changes in permeability in a high K medium, in the presence of acetylcholine and a pH of 8, under which conditions it produced maximum inhibition of cholinesterase.[1]

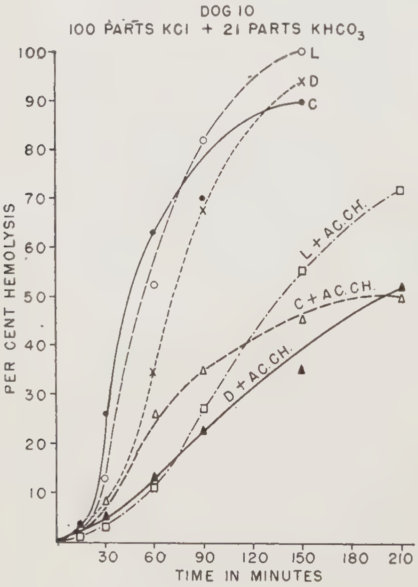

FIG. 2.

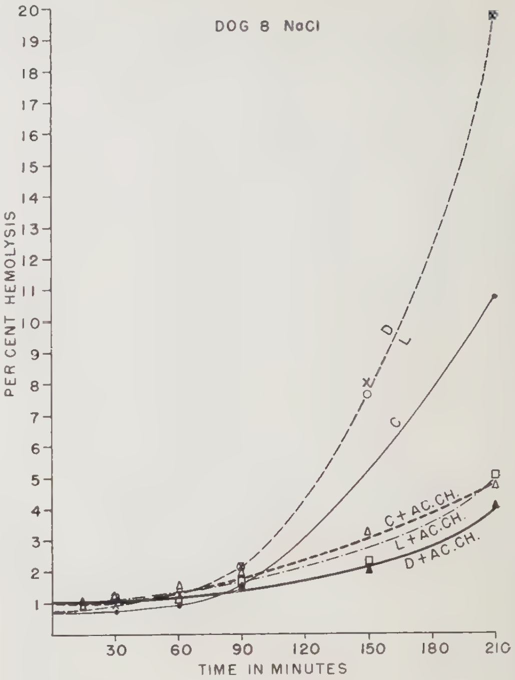

FIG. 3.

Investigations of ionic transfer between erythrocytes and the medium in the presence of cholinesterase inhibitors are under way.

*Summary.* The theory that the acetylcholine-cholinesterase system is concerned with membrane permeability is further substantiated by the finding that the L-isomer of isoamidone which has a greater inhibitory action on cholinesterase than has the D-isomer also changes permeability of dog erythrocytes to a different degree than does the D-isomer.

The conditions under which the L has a greater effect than the D-isomer on permeability are a medium containing a high proportion of K ions relative to Na ions.

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# 17130. Interrelationship of Vitamin $B_{12}$ and Choline.* I. Effect on Hemorrhagic Kidney Syndrome in the Rat.

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Following the isolation of vitamin $B_{12}$ by Rickes and co-workers,[1] several investigators[2-4] have reported data indicating the identity or close relation of this vitamin with the "animal protein factor." Studies by Bird, Rubin, and Groschke[5] have indicated that methionine can function as a partial substitute for the "animal protein factor" in soybean protein diets for chicks. In view of the relationship between methionine and choline, it seemed of interest to determine whether vitamin $B_{12}$ might exert a sparing action on the choline requirement of the rat.

It is the purpose of this paper to report that the incidence and the severity of renal hemorrhage in weanling rats receiving a diet low in choline and methionine were significantly decreased by supplementing the diet with vitamin $B_{12}$ in concentrate or crystalline form.

*Experimental.* Weanling rats of the AES (Alabama Experiment Station) strain weighing 40-50 g were placed in individual cages and uniformly grouped with respect to number, weight, sex and litter. Feed and water were supplied *ad libitum*. The basal diet contained extracted peanut meal 30,[6] sucrose 39.5, extracted casein 6,[7] salt mixture[7] (undried) 4.4, L-cystine 0.1, cod liver oil 1, lard 19. Vitamins were added, mg/kg of diet, as follows: thiamine 2, pyridoxine 2, riboflavin 4, calcium pantothenate 10, niacin 20, i-inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, and menadione 5. The total methionine and choline content of the diet was .3% and .007% respectively.

On this basal diet without added choline or methionine, a 100% incidence of fatal kidney hemorrhage occurs in weanling rats of the AES strain in 2 weeks or less. In the present experiments, various sub-protective levels of choline or methionine were added to the basal diet. The effect of adding vitamin $B_{12}$ in concentrate or crystalline form to the diet for each of the treatments was then determined. In one experiment the effect of adding vitamin $B_{12}$ concentrate to the basal diet supplemented with an adequate protective level of choline (.2%) was determined. Except in the latter experiment, all rats were necropsied after death or at the end of a 14-day experimental period, and the kidneys were carefully examined for gross pathological lesions.

The results on the effects of vitamin $B_{12}$ with sub-protective levels of choline are shown in Table I. At levels of .04% and .06% of choline chloride without vitamin $B_{12}$, the

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station. This work was supported in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. It was also aided by donations of concentrates and crystalline vitamin $B_{12}$ and other vitamins by Merck and Co., and of folic acid by Lederle Laboratories. These data were presented in part at the Birmingham meeting of the Southern Section, Society for Experimental Biology and Medicine, February 15, 1949, and at the Detroit meeting, Fed. Am. Soc. Exp. Biol., April 20, 1949.

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[5] Bird, H. R., Rubin, M., and Groschke, A. C., *J. Nutrition*, 1947, **33**, 319.

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[7] Salmon, W. D., *J. Nutrition*, 1947, **33**, 155.

TABLE I.
Choline-Sparing Action of Vitamin $B_{12}$ for Protection Against Hemorrhagic Kidneys.

| Dietary supplement | | | | | |
|---|---|---|---|---|---|
| Choline Cl., % | Source of vitamin $B_{12}$, ≅ μg/kg diet | No. of rats | Mortality | Avg wt gain of survivors g/2 wks | Incidence of renal damage, % |
| .04 | 0 | 25 | 24 | 19 | 100 |
| .05 | 0 | 4 | 0 | 36 | 75 |
| .06 | 0 | 16 | 0 | 44 | 69 |
| .20 | 0 | 4 | 0 | 57 (130)* | 0 |
| .02 | Conc. No. 1† ≅ 30 | 4 | 50 | 50 | 75 |
| .03 | ” ” 1 ≅ 60 | 4 | 0 | 38 | 25 |
| .03 | ” ” 1 ≅ 30 | 4 | 25 | 39 | 50 |
| .04 | ” ” 1 ≅ 30 | 21 | 9.5 | 55 | 38 |
| .05 | ” ” 1 ≅ 30 | 4 | 0 | 45 | 25 |
| .06 | ” ” 1 ≅ 30 | 10 | 0 | 57 | 20‡ |
| .20 | ” ” 1 ≅ 30 | 4 | 0 | 58 (132)* | 0 |

* Avg wt gain at 4 weeks.
† Merck & Co., Inc.—charcoal adsorbate No. 8R 5704.
‡ Very slight renal damage in 2 of the 10 rats.

TABLE II.
Choline-Sparing Action of Vitamin $B_{12}$ for Protection Against Hemorrhagic Kidneys.

| Dietary supplement | | | | | |
|---|---|---|---|---|---|
| Choline Cl., % | Source of vitamin $B_{12}$, ≅ μg/kg diet | No. of rats | Mortality, % | Avg wt gain of survivors, g/2 wks | Incidence of renal damage % |
| .04 | None | 4 | 25 | 14 | 100 |
| .04 | Crystalline $B_{12}$, 30 | 4 | 25 | 43 | 50 |
| .04 | Conc. No. 1 ≅ 30 | 4 | 25 | 39 | 50 |

TABLE III.
Methionine-Sparing Action of Vitamin $B_{12}$ Concentrate for Protection Against Hemorrhagic Kidneys.

| Dietary supplement | | | | | |
|---|---|---|---|---|---|
| DL-methionine, % | Source of vitamin $B_{12}$, ≅ μg/kg diet | No. of rats | Mortality, % | Avg wt gain of survivors, g/2 wks | Incidence of renal damage, % |
| .150 | 0 | 4 | 75 | 46 | 100 |
| .192 | 0 | 8 | 25 | 27 | 100 |
| .150 | Conc. No. 1 ≅ 30 | 4 | 25 | 57 | 25 |
| .192 | ” ” 1 ≅ 30 | 8 | 0 | 60 | 12 |

incidence of kidney damage was 100% and 69%, respectively. The inclusion of a charcoal adsorbate to supply 30 μg of vitamin $B_{12}$ per kg of diet to the above treatments reduced the incidence of kidney damage to 38% and 20%, respectively. It appeared that .03% of choline with vitamin $B_{12}$ was as effective in preventing kidney damage as .06% of choline without the vitamin.

In general, the severity of renal damage in the rats receiving vitamin $B_{12}$ was relatively mild in comparison with the damage in the control rats not receiving the vitamin. Only 2 of 21 rats receiving .04% of choline with vitamin $B_{12}$ concentrate died; the average weight gain of the survivors for the 2-week period was 55 g. Of the 25 rats receiving .04% of choline chloride without vitamin $B_{12}$, 6 died and the average weight gain of the survivors for the 2-week period was only 19 g. The increased gain of the rats receiving the vitamin $B_{12}$ preparations was in all probability a direct result of the protective action of the vitamin. It certainly emphasizes the protective effect of the vitamin because in the absence of such protection the increased gain would have increased the incidence and the severity of the kidney lesions.

That vitamin $B_{12}$ was the active principle in the concentrates used is indicated by the results shown in Table II. In this series crystalline vitamin $B_{12}$ was compared with the charcoal adsorbate at the .04% level of choline chloride.

Vitamin $B_{12}$ likewise increased the efficiency of methionine for the prevention of kidney damage (Table III). Supplementary DL-methionine was added to the basal diet at levels of .15% and .192%. The incidence of kidney damage was 100% at both levels. However, when the diet was further supplemented with vitamin $B_{12}$, the incidence of kidney damage decreased to 25% at the .15% level of methionine and to 12% at the .192% level.

The results when vitamin $B_{12}$ was added to the diet with an adequate protective level of choline are shown in Table I. There was no effect on weight gains or appearance of the rats at the end of the second or fourth week. Rats in this laboratory have consistently made normal growth on this diet when adequate choline was added without either folic acid or vitamin $B_{12}$. When .2% choline chloride was added to the basal diet the need for supplementary vitamin $B_{12}$ was eliminated. It thus appears that dietary choline has a significant sparing action on vitamin $B_{12}$.

*Discussion.* The results of these experiments show that vitamin $B_{12}$ decreases the dietary choline or methionine required for protection against the hemorrhagic kidney syndrome in rats. It appears that, under the conditions of these experiments, about one-half of the choline required for protection against this syndrome can be replaced by 30 $\mu$g of vitamin $B_{12}$ per kg of diet. Moreover, the results of one experiment indicate that the need for vitamin $B_{12}$ is markedly decreased by the inclusion of adequate choline in the diet.

Thus, there is established the existence of an interrelationship between vitamin $B_{12}$ and choline or methionine when the latter functions as a choline precursor. This interrelationship assumes added significance in view of the apparent importance of these factors in the maintenance of normal hemoglobin levels in humans as well as in experimental animals. Vitamin $B_{12}$ has been shown by West[8] to be effective in the treatment of Addisonian pernicious anemia. Spies and his collaborators[9] have recently reported satisfactory hematologic responses to vitamin $B_{12}$ therapy in persons suffering from pernicious anemia, nutritional macrocytic anemia, or tropical sprue. They also found the vitamin to be effective in relieving the subacute combined nervous system degeneration associated with pernicious anemia.

Engel[6] has reported anemia and edema in rats on low-protein diets that were deficient in choline. The addition of choline chloride prevented both the anemia and the edema. McKibbin, Thayer and Stare[10] have reported lowered hemoglobin values in dogs receiving diets deficient in choline. We have observed severe anemia in dogs on a choline-deficient diet similar to the diet used in the experiments herein reported.[11]

Choline has been used for the treatment of anemia in humans with variable results. Moosnick, Schleicher and Peterson[12] have reported successful treatment with choline of a case of pernicious anemia that was refractory to parenteral liver therapy. Davis and Brown[13] have reported hematologic responses to choline therapy in 4 case of pernicious anemia and one case of megaloblastic anemia of pregnancy; choline was ineffective, however, in one other case of pernicious anemia and 4 other cases of megaloblastic anemia.

The demonstration of a specific interrelationship of vitamin $B_{12}$ and choline in protecting against the hemorrhagic kidney syndrome in rats, may suggest that the use of choline as a supplementary aid in the treatment of various types of anemia should be further explored. Some of the clinical results referred to above indicate that it may be of particular value in cases complicated with liver damage. Further investigations on the nutritional implications of this interrelationship are underway in this laboratory.

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*Summary.* 1. The incidence and severity of renal injury in weanling rats fed diets low in choline and methionine were markedly decreased by supplementing the diet with a vitamin $B_{12}$ concentrate or crystalline vitamin $B_{12}$.

2. Under the conditions of these experiments, 30 $\mu$g of vitamin $B_{12}$ per kg of diet could replace about one-half of the supplementary choline or methionine required for protection against kidney damage.

3. When sub-protective levels of choline were fed, the addition of vitamin $B_{12}$ caused a significant increase in weight gain. However, when an adequate protective level of choline was fed, no increase in weight gain was obtained from the addition of the vitamin.

4. The results established the existence of an interrelationship between vitamin $B_{12}$, and choline or methionine.

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## 17131. Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration.

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An inquiry has been made into conditions which may influence the viability of 1x1 cm areas of biopsied rabbit skin during refrigeration at 0° and 6-8°C. Since the availability of oxygen, as well as the nature of the storage medium, had an important influence on the preservation of viability at these two temperatures, these relationships may be of interest to those wishing to store or ship tissues for surgical and other purposes.

Tissues separated from the circulation rapidly become anoxemic and necrotic at room or body temperatures. This condition may be prevented in uterine or intestinal strips by oxygenation or by chilling.[1,2] The survival of ligated limbs[3] and of the cells in whole embryos[4,5] or organs[5,6] is optimal (among the widely spaced temperatures which have been studied) at 0°. At this temperature respiration is minimal, while oxygen solubility in water is twice that at 30°.

Though Lambert,[7] Carrel,[8] and Hetherington and Craig[5] found 0 to 7° favorable for preserving the small masses of crowded cells in embryonic tissue fragments, there is considerable evidence that the thin perimeter of migrating and dividing cells in established tissue cultures has maximal longevity around 30°[9,10] and are unable to re-establish growth after refrigeration for a few days.[9,11-13] Upon considering the fact that large tissues are

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[2] Nolf, P., *Arch. Internat. de physiol.*, 1928, **30**, 315.

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[7] Lambert, R. A., *J. Exp. Med.*, 1913, **18**, 406.

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[11] Carpenter, E., *J. Exp. Zool.*, 1945, **98**, 79.

[12] Fischer, A., *Arch. f. exp. zellf.*, 1926, **2**, 303.

[13] Hanks, J. H., unpublished.

killed more rapidly at higher temperatures and small groups of thinly spread cells at low temperatures, it seemed not unlikely that one of the common denominators might be a question of oxygen supply and demand.

*Methods.* After shaving and scrubbing, the chosen area of rabbit skin was covered for 2 minutes with a wet pack of tincture of iodine diluted 1:2 in water, and again scrubbed with alcohol. The strips of biopsied skin, either full- or half-skin depth were cut into 1x1 cm pieces. One piece of skin was explanted immediately and the others (usually 8) were stored.

The tissues were stored in two types of containers: a) 13 mm test tubes with just sufficient (0.2 cc) mineral oil or 10% rabbit serum to cover tissues, and also in such tubes filled with 9 cc of these fluids in order to replace the atmosphere, or b) in 6 cc screw cap vials suitable for mailing. These vials were packed approximately ¾ full of washed Pyrex glass wool and a second (removable) plug was added to fill the remaining space. One cubic centimeter of sterile medium moistened the entire surface of the fibers by capillarity, leaving about 4.5 cc of air space, while 5.5 cc fluid completely displaced the atmosphere. Penicillin and streptomycin at 100 units per ml. and sulfadiazine at 20 mg% were at times included in the storage media.

*Tissue culture reagents.* 1. Balanced salt solution (BSS): One liter of concentrated stock stored at room temperature with 4 cc chloroform: NaCl, 80 g; KCl, 4.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2$, 1.4 g (dissolved separately); $Na_2HPO_4 \cdot 2H_2O$, 0.6 g (1.5 mg of $Na_2HPO_4 \cdot 12H_2O$); $KH_2PO_4$, 0.6 g; glucose, 10.0 g. To this is added 100 cc of phenol red 0.2%. This stock solution was diluted 1:10 (Ca = 5 mg %, P = 2.4 %) and autoclaved in 20 cc amounts in screw cap Pyrex bottles. One half cubic centimeter of autoclaved 1.4% (isotonic) $NaHCO_3$ was added to each bottle and the solution stored in the refrigerator for $CO_2$ equilibration to pH 7.6 before final tightening of the caps.

2. *Chicken plasma* (*CH*) Cock blood was drawn into a 10 cc syringe containing 0.2 cc of 10% sodium citrate, centrifuged twice and stored in 1 to 2 cc amounts in plain glass tubes at –20°C.

3. *Beef embryo juice* ($EM_{50}$): Three- to 5-inch beef embryos were emulsified in a Waring blendor for 1.5 minutes with an equal volume of BSS. The suspension was centrifuged at top speed for 30 minutes and the supernate respun in cotton balltubes.

4. Rabbit serum fresh, or frozen at –20°C.

Efficient sterility tests were provided by having a small fluff or ball of cotton in all tubes used for the second centrifugation. Clean separation of the supernate from the sediment was assured, while approximately 99% of the possible contaminating organisms remained below the packed cotton. Brewer's medium was pipetted onto the cotton and the sediment to a depth of approximately 3 inches and the whole incubated at 31° for one week.

*Tissue culture methods.* Both freshly biopsied and stored skin* were sampled for viability after being cut into 2x2 mm fragments in Brewer's medium, which was pipetted off for sterility control. Twelve of the fragments from each piece of skin were selected at random, 3 being transferred to each of 4 tubes previously coated with one drop of chicken plasma. After orientation of the explants and mixing the three drops of medium (see liquid phase) with the plasma, the tubes were laid horizontally for coagulation. One cubic centimeter of liquid phase (rabbit serum 20%, embryo juice 2%, penicillin and streptomycin 100 units each and sulfadiazine 20 mg %) was added to each tube prior to stoppering and slanting for incubation at 37° for one week.

At the end of one week, data were collected on the proportion of explants showing positive growth and on the area of new growth around the explants. Epithelial and fibrocytic outgrowth was distinguished and each rated on a scale of 1 to 4 plus, 1+ being assigned to explants with sufficient cells to surround only one of the four sides and 4+ to those completely surrounded.

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* The removal of mineral oil from stored tissues by shaking them in several replacements of ethyl ether did not influence their viability.

TABLE I.
Survival of Biopsied Skin at Low Temperatures.

| Exp. No. | Type of biopsy | Explanted immediately | | | | Storage | | Survival at 1 week | | | | Survival at 2 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % Pos. | New area (mm²) | Cell* rating E | Cell* rating F | Temp. °C | Medium | % Pos. | New area (mm²) | Cell rating E | Cell rating F | % Pos. | New area (mm²) | Cell rating E | Cell rating F |
| 1 | Full depth | 100 | 16 | 2.3 | 3.4 | 8 | Min. oil full | 67 | 19 | 1.0 | 1.5 | 0 | | | |
| | | | | | | | Min. oil 0.2 cc | 100 | 35 | 1.3 | 3.7 | 8 | 0 | | |
| | | | | | | | $RS_{10}$† full | 100 | 8 | 1.3 | 2.9 | 58 | 1.3 | .2 | .3 |
| | | | | | | | $RS_{10}$ 0.2 cc | 100 | 35 | 1.5 | 3.2 | 100 | 35.0 | 2.8 | 3.7 |
| 2 | Full depth | 100 | 24 | 0.5 | 3.1 | 0 | Min. oil full | 42 | 2 | 0.5 | 1.4 | 0 | | | |
| | | | | | | | Min. oil 1 cc | 0 | | | | 0 | | | |
| | | | | | | | $RS_{10}$ full | 92 | 9 | 1.0 | 2.7 | 17 | 0 | 0 | 0.05 |
| | | | | | | | $RS_{10}$ 1 cc | 83 | 6 | 1.1 | 2.3 | 8 | 0 | 0 | 0.01 |
| 3 | Full depth | 100 | 16 | 0.1 | 3.6 | 0 | $RS_{10}$ full | 92 | 15 | 0.3 | 3.0 | | | | |
| | | | | | | | R blood (1 cm) | 92 | 15 | 0.1 | 2.3 | | | | |
| | Tiersch graft | 100 | 16 | 0.4 | 3.2 | 0 | $RS_{10}$ full | 83 | 8 | 0 | 1.9 | | | | |
| | | | | | | | $RS_{100}$ full | 100 | 16 | 0 | 2.8 | | | | |
| | | | | | | | R blood (1 cm) | 100 | 16 | 0 | 2.7 | | | | |
| | | | | | | | R blood (3 cm) | 67 | 6 | 0 | 0.8 | | | | |

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* Epithelial (E) and fibrocytic (F) outgrowth from each explant were rated on the 4+ scale. Cell ratings are average values from each group of 12 cultures. Since one side of the explants was covered with epidermis, 1+ or 2+ tends to be a maximal value for E and 3+ a maximal value for F during early outgrowth.

† Rabbit serum 10%, $RS_{100}$ = Rabbit serum 100%.

*Results.* Representative results are summarized in Table I. During refrigeration at 6-8°, the exclusion of atmosphere was always detrimental (see first experiment). In tubes or vials filled with mineral oil the blood in the vessels became blue, and the viability declined in less than one week. No cells could be cultivated after two weeks. In tubes filled with rabbit serum 10% the viability declined steadily over the two week period. The pH indicator in the upper stratum of the liquid retained its original color while the fluid nearer the tissues became strongly acidified within one week.

With just enough oil to cover the skin and prevent evaporation, the blood in the vessels remained red. Viability was maintained at a high level for one week, but declined sharply during the second week. In shallow layers of rabbit serum 10% the pH fell to 6.8 or 7.0 within one week but in spite of the small reserve of medium and of buffer the viability did not decline within two weeks.

Preservation of viability at 0° proved to be far less satisfactory in any medium employed, and particularly in the presence of atmosphere (see second experiment). No biopsies survived for one week in 0.2 to 1.0 cc mineral oil. In tubes filled with mineral oil to exclude atmosphere, the results were strikingly more favorable, since approximately 50% of the cells remained alive during the first week. No cells survived after preservation in oil for two weeks. In rabbit serum 10% of the cells were more adequately supported or protected, and the results again revealed the same relationship with respect to the influence of atmosphere. In the shallower layers of medium the viability at the end of one or two weeks was only 50 to 70% of that in tubes which had been completely filled with liquid.

Since blood provides a mass of cells capable of competing for the available oxygen, this menstruum was also employed in certain of the experiments at 0°. The third experiment includes data on both Tiersch graft and full-depth biopsies removed from the same rabbit and stored at 0° for one week. It may be seen that a blood coagulum of only one centimeter in depth proved as effective as completely filling the tubes with rabbit serum 10% or 100%. Greater depths of blood (*e.g.* 3 cm) were not superior, but were sometimes disadvantageous.

The poor migration of epithelial cells in certain experiments was due to incomplete removal of the iodine used for skin sterilization (see next paper).

*Discussion and summary.* Although the storage of tissues at low temperature has long been recognized as a convenient means of preserving viability for relatively short periods of time, the potential advantages of refrigeration require further investigation of the nutrient materials needed to sustain metabolism and of the conditions which permit conservation of the essential enzyme systems. From the present comparison of survival in mineral oil and in dilute serum, it is evident that the usefulness of vaseline,[8,14] pliofilm[15] or moist gauze[16] as storage vehicles may be questioned. These earlier methods provide no nutrient materials and make no provision for leaching out or buffering the acids which are produced in the living tissues.

No simple rules can be stated for the control of the oxygen-temperature relationship, since it will be modified by the depth of the medium employed and by the form and mass of the tissue, and must be determined for each type and volume of tissue to be stored. For the shipping of tissues in iced containers the use of a blood coagulum may be regarded on the one hand as a convenient means of increasing the cell mass or, on the other, of providing the necessary exclusion of atmospheric oxygen.

Since blood does not permit observation of pH changes as an index of the need to replace nutrient fluids, and the blood cells must inevitably contribute to acidification and exhaustion of the medium, this menstruum is considered less desirable than a non-cellular fluid for the "banking" of tissues during hospital storage for surgical purposes. Furthermore, it is evident that during refrigeration at 6-8° any increases in nutrient and buffering

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[14] deMartigny, F., *Congr. franc. chir.*, 1913, **26**, 252.

[15] Webster, J. P., *Am. J. Surg.*, 1944, **120**, 431.

[16] Matthews, D. N., *Lancet*, 1945, **1**, 775.

fluids should be made horizontally, *i.e.*, without restricting the availability of atmospheric oxygen.

A study by the surgical staff of the Children's Hospital of the preservation of aortic segments for surgical repair of large blood vessels has produced results in accordance with these principles. Their publication[17] describes the conditions under which blood vessels may be maintained in 10% homologous serum for as long as 50 days.

In conclusion, 10% serum is superior to mineral oil as a refrigeration menstruum because it provides nutrient and dilutes or buffers the acids which result from metabolism. At 6-8° oxygen is required to support metabolism, while at 0° viability is extended by completely filling the storage tubes with liquid or by covering the tissue with a blood coagulum.

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[17] Peirce, E. C., Gross, R. E., Bill, A. H., and Merrill, K., *Ann. Surg.*, 1949, **129**, 333.

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## 17132. Sterilization of Skin.

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It is frequently stated that in the strict bacteriological sense it is impossible to sterilize skin. This concept apparently arises from the use of methods which do not insure that an appropriate disinfectant is applied in active form for an adequate interval of time. Since the rich and complex nutrients used for cell cultivation provide an excellent pabulum for a variety of microorganisms, long experience in sterilization of leprous and normal skin prior to biopsy for tissue culture induces us to present evidence that skin sterilization is not a difficult problem.

Early attempts to sterilize well scrubbed skin by painting with tincture of iodine were at times successful and at times failures. The inconstancy of the results suggested the desirability of controlling the action of iodine with respect to concentration and time of action. By covering the area to be biopsied with a heavy patch of fabric, dripping iodine solution (tincture of iodine 1:2 in 70% alcohol by weight) on this patch until it was saturated, and then removing the patch and recleaning with alcohol after an interval of 5 minutes, the occurrence of scattered contamination was terminated. During the next several years it was noted that epithelial cells rarely if ever migrated from fragments explanted from the papillary or uppermost layer of the skin, while abundant epithelial cells appeared around fragments explanted from the deeper or reticular layer of the skin.

Upon the first occasion when a biopsy was taken after painting the skin with 2% Mercurochrome in Duponol as a wetting agent, good epithelial growth around papillary explants appeared for the first time in our experience. Nevertheless, one after another of the culture tubes gave evidence of contamination by staphylococci until 40% of the cultures had been lost. The period of applying the iodine (1:2) patch was next shortened to 2 minutes, without trouble from contamination and with the result that the superficial layer of skin produced moderate to excellent epithelial migration.

In order to inquire whether other disinfectants might be as effective as dilute iodine under these conditions and possibly less inhibitory to epithelial migration *in vitro*, three strips of skin were removed from the same individual simultaneously, one having been prepared by a two minute dressing with tincture of iodine 1:2, the second with Zephiran 1:1000, and the third with 2% aqueous Mer-

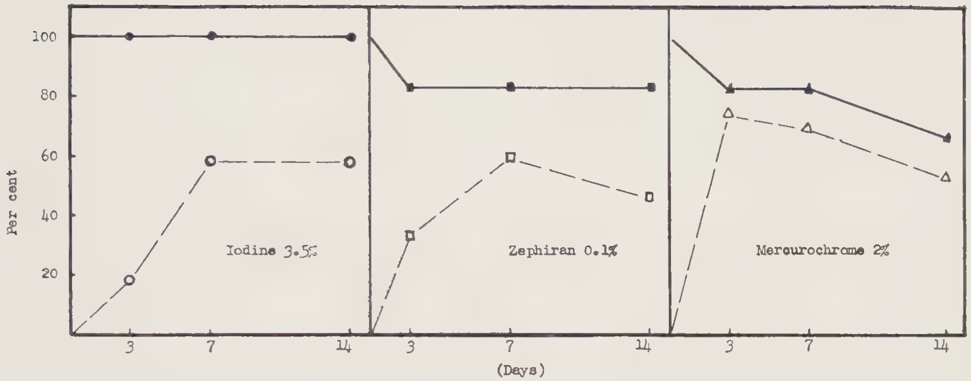

FIG. 1.

The proportion of explants free of contamination (solid symbols) and the proportion positive for epithelial cells (open symbols). Thirty-three cultures in each group.

curochrome. The results (illustrated in Fig. 1) were the usual ones after iodine patching, namely, no contamination, but appreciable inhibition of epithelial migration. With Zephiran 17% of the cultures were lost by contamination within the first 3 days, while migration of epithelium was not significantly improved. The Mercurochrome treatment was definitely less inhibitory to epithelial cells, but did not afford an increase in the number of useful cultures, due to a 33% loss by contamination. Since fibroblasts grew from the skin with equal readiness irrespective of the disinfecting agent, the dilute iodine produced the greatest proportion of positive and usable cell cultures.

Lest the aqueous solution of Mercurochrome might have given results poorer than those obtainable in the presence of a wetting agent, the effectiveness of Mercurochrome 2% in Duponol was compared against tincture of iodine diluted 1:4 in 70% alcohol, each being applied as a moist pack for 2 minutes prior to the biopsy of rat skin. During the first two weeks of cell cultivation *in vitro,* the incidence of contamination proved to be 20% from the Mercurochrome biopsy and 4% following the use of the 1:4 dilution of iodine.

Subsequent work with tincture of iodine 1:4 in 70% alcohol, with application for five minutes, indicates complete reliability, but that the same care must be exercised in its removal as in the case of the 1:2 solution. Further experience is required to evaluate the routine effectiveness of the 1:4 dilution during application for only two minutes.

*Discussion.* In Price's excellent report on disinfection of skin,[1] it is emphasized that the germicidal action of 7% iodine tincture practically ceases upon drying. It is shown that 2% of iodine in alcohol 70% by weight evaporates more slowly and avoids the objectionable irritating effects of the 7% tincture. This 2% solution was more effective following a single surface application than any other class of disinfectant studied. In other sections of the report the time factor is repeatedly emphasized. The results reported represent an application of the principles elucidated by Price.

It is not improbable that a mixture of penicillin, streptomycin and sulfadiazine for skin treatment, and in the cell culture media would provide a more appropriate measure of optimal epithelial migration than any of the disinfectants used. In view of the long period of cell cultivation required in leprosy research, this comparison has been impractical for two reasons: (a) the danger of inhibiting the infectious agent, and (b) the inability of such a mixture to inhibit the development of fungi from the skin explants.

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[1] Price, P. B., *J.A.M.A.*, 1938, **111**, 1939.

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## 17133. Interrelationship of Vitamin $B_{12}$ and Choline. II. Effect on Growth of the Chick.*

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In the accompanying paper it was reported that the severity and incidence of renal hemorrhage in weanling rats fed diets low in choline and methionine were significantly decreased by supplementing the diet with vitamin $B_{12}$.[1] In concurrent experiments the interrelationship of the vitamin $B_{12}$ and choline requirements for growth of the chick was studied.

*Experimental.* Day-old Leghorn chicks of the A.P.I. strain were divided into 3 weight classes ranging from 34 to 42 g. The selected chicks were then distributed, 8 to 12 per group, so that the group average was about 38 g. The chicks were wing-banded and housed in standard commercial, electrically heated batteries, which were equipped with raised wire floors. Feed and water were supplied *ad libitum* and the chicks were weighed weekly. The basal diet was composed of extracted peanut meal 30,[2] sucrose 36.9, extracted casein 6,[3] L-cystine .1, cod liver oil 1, lard 19, $Ca_3(PO_4)_2$ 2.87, $K_2HPO_4$ 1.442, $CaCO_3$ .903, $MgSO_4 \cdot 7H_2O$ .840, NaCl .70, Fe Citrate .182, $MnSO_4 \cdot 4H_2O$ .035, $ZnCO_3$ .014, $CuSO_4 \cdot 5H_2O$ .007, and KI .007. Vitamin supplements, mg/kg diet, were as follows: thiamine 3, pyridoxine 4, riboflavin 6, calcium pantothenate 20, niacin 50, inositol 1000, folic acid 1, biotin .25, alpha tocopherol 25, alpha tocopherol acetate 25, and menadione 5. The diets were mixed at 10-day intervals and stored at 1°-5°C. The basal diet furnished a maximum of .007% choline and .3% methionine. Preliminary studies had established that supplementation of this basal diet with .6% choline chloride was necessary for maximum growth.

A summary of four experiments is given in Table I. Concentrates of vitamin $B_{12}$ were added to the basal diet at the expense of sucrose and the choline supplement was reduced in the amount that was furnished by the concentrates. Choline was determined by the procedure of Engel,[4] modified by washing the choline reineckate precipitate with cold N-propyl alcohol in place of methyl alcohol. Vitamin $B_{12}$ concentrates Nos. 1 and 3 were a charcoal and fullers earth adsorbate, respectively. The crystalline vitamin $B_{12}$ was in the form of a solution containing 25 $\mu$g/ml.

When the basal diet was supplemented with .6% choline chloride, the addition of vitamin $B_{12}$ concentrates Nos. 1 or 3 at a level to supply 30 $\mu$g vitamin $B_{12}$ per kg diet caused an average increase in weight gain of 24 to 28 g in 2 weeks, or 59 to 66 g in 4 weeks. The feeding of the concentrates to supply 15 $\mu$g vitamin $B_{12}$ per kg diet resulted in a weight increment of 52 to 62 g in 4 weeks.

When the choline supplement was reduced to .2%, the addition of vitamin $B_{12}$ concentrate caused an increase in weight gain at 2 weeks of 32 g and 100 g in 4 weeks. At the .1% level of choline chloride supplement the greatest increase in weight gain due to the vitamin $B_{12}$ concentrate was observed. The weight increment was 63 g at 2 weeks and 186 g at 4 weks. The total weight of these birds was as good as that obtained when the

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[1] Schaefer, A. E., Salmon, W. D., and Strength, D. R., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 193.

[2] Engel, R. W., *J. Nutrition*, 1948, **36**, 739.

[3] Salmon, W. D., *J. Nutrition*, 1947, **33**, 155.

[4] Engel, R. W., *J. Biol. Chem.*, 1942, **144**, 701.

TABLE I.

| Exp. No. | Dietary supplement: Choline chloride % | Dietary supplement: Source of vitamin $B_{12} \cong \mu g\ B_{12}$/kg diet | No. of chicks | Avg wt gain g/chick: 2 wk | Avg wt gain g/chick: 4 wk |
|---|---|---|---|---|---|
| 4 | .05 | 0 | 11 | 20 | 46 |
| 3, 4 | .1 | 0 | 23 | 27 | 60 |
| 2 | .2 | 0 | 12 | 31 | 95 |
| 1, 2, 3, 4 | .6 | 0 | 47 | 54 | 154 |
| 4 | .05 | $B_{12}$ conc. No. 1 $\cong$ 15 | 11 | 41 | 150 |
| 3 | .1 | ” ” ” 1 $\cong$ 30 | 12 | 90 | 246 |
| 4 | .1 | ” ” ” 1 $\cong$ 15 | 11 | 72 | 262 |
| 1, 2, 3 | .2 | ” ” ” 1 $\cong$ 30 | 34 | 63 | 195 |
| 1, 2, 3, 4 | .6 | ” ” ” 1 and 3 $\cong$ 30 | 59 | 79 | 218 |
| 1, 3 | .6 | ” ” ” 1 and 3 $\cong$ 15 | 22 | 78 | 210 |

diet was supplemented with .6% choline chloride plus vitamin $B_{12}$ concentrate (Fig. 1).

When the choline supplement was .05%, the increase in weight resulting from the addition of vitamin $B_{12}$ concentrate was less than that observed in the groups receiving .1% and .2% choline chloride. This indicates that this level of vitamin $B_{12}$ cannot replace the entire dietary choline required by the chick but that a minimum level of choline must be present.

That vitamin $B_{12}$ was the active principle in the concentrates is indicated by the data in Table II. Pure vitamin $B_{12}$ fed at a level of 15 μg per kg of diet caused an average increase in weight gain of 14 g at 1 week and 40 g in 2 weeks as compared to 14 g at 1 week and 45 g in 2 weeks for the group receiving the vitamin $B_{12}$ concentrate.

*Discussion.* The choline requirement for normal growth of chicks fed the basal diet without a source of vitamin $B_{12}$ was .6% choline chloride as compared to .1% when vitamin $B_{12}$ was added to the diet. This interrelationship of vitamin $B_{12}$ and choline for the chick is in agreement with the results obtained with rats.[1]

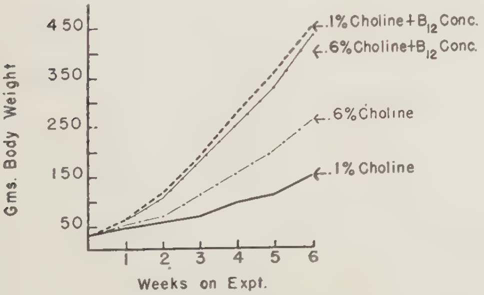

FIG. 1.

Sparing action of vitamin $B_{12}$ concentrate on the choline requirement of chicks. (12 chicks per group).

The chicks used in these experiments were progeny of hens from a strain that had been bred for high livability. The breeding flock is on commercial breeding ration, which supports good egg production. The progeny of these hens have shown a very uniform growth response to the addition of vitamin $B_{12}$. Not only was this growth increment uniform but it was also much greater than that reported by other investigators using other types of diets. For example, when the basal diet was supplemented with .1% choline chloride plus a source of vitamin $B_{12} \cong 30$ μg per kg of diet, the growth increment was 63 g at 2 weeks and 186 g at 4 weeks. This increment is considerably greater than that reported by the following workers: Lillie, *et al.*,[5] 34 to 60.3 g increase in body weight from the second to the fourth week; Nichol, *et al.*,[6] 79 to 88 g for a similar period, and Ott, *et al.*[7] of 20 to 46 g at 17 days.

These observations of the influence of choline level on the vitamin $B_{12}$ requirement may be useful when procedures for a chick assay of vitamin $B_{12}$ are being standardized. The data presented in Fig. 1 indicate that vitamin $B_{12}$ can be assayed more effectively if

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[5] Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **176**, 1477.

[6] Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 40.

[7] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, **174**, 1047.

TABLE II.
Crystalline Vitamin $B_{12}$ Compared with Vitamin $B_{12}$ Concentrate.

| Dietary supplement | | No. of chicks | Avg wt gain g/chick | |
|---|---|---|---|---|
| Choline chloride % | Source of vitamin $B_{12}$ $\cong \mu g$ per kg diet | | 7 days | 14 days |
| .1 | None | 11 | 16 | 27 |
| .1 | Crystalline vit. $B_{12} \cong 15$ | 7 | 30 | 67 |
| .1 | $B_{12}$ conc. No. 1 $\cong 15$ | 11 | 30 | 72 |

the basal diet is supplemented with .1% choline chloride than when it is supplemented with .6%. It would be of interest to determine whether the feeding of a low-choline diet to hens would increase the requirement for the "animal protein factor" in the progeny, similar to that noted when the hens were restricted to a diet low in the "animal protein factor".[5,6]

The interesting interrelationship of vitamin $B_{12}$, choline, and methionine is being studied further in this laboratory.

*Summary.* 1. Vit. $B_{12}$ was fed to Leghorn chicks receiving a choline-low basal diet supplemented with .6%, .2%, .1%, and .05% choline chloride. The 14-day increment in weight gain over the controls that did not receive vitamin $B_{12}$ was 26 g, 32 g, 63 g, and 21 g, respectively.

2. Crystalline vit. $B_{12}$ fed at a level of 15 $\mu g$ per kg of diet and at a choline level of .1% was as effective as vit. $B_{12}$ concentrate in a 2-week feeding period.

3. The choline requirement of chicks under the conditions of these experiments was markedly reduced by the supplementation of the diet with vit. $B_{12}$.

4. It appears that dietary choline has a significant sparing action on vit. $B_{12}$.

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## 17134. An Oxygenator with Increased Capacity: Multiple Vertical Revolving Cylinders.*

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The vertical revolving cylinder has been shown to be an effective oxygenator for blood.[1,2] Its efficiency as an oxygenator is probably increased by making the cylinder shorter in relation to its diameter.[3] These data were applied in the construction of another oxygenator with approximately eight times the capacity of the oxygenator previously studied.

*Experimental.* Eight concentric cylinders (Fig. 1, C) 21 cm in height, ranging from 27.5 cm to 17.0 cm in diameter are mounted so that the lower edges of the cylinders are approximately one centimeter above the inner surface of a cone (f). The surface area is 14,500 $cm^2$. Blood is delivered through individual spouts (b) onto the inner surface of each cylinder, drops off of its lower edge onto the inner surface of the cone, flows into the vertical shaft (t), runs down the inner surface of the shaft, and collected in the stationary cup (h) beneath. A 94% $O_2$ and

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* Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

[1] Gibbon, J. H., Jr., *J. Lab. and Clin. Med.*, 1939, **24**, 1192.

[2] Gibbon, J. H., Jr., and Kraul, C. W., *J. Lab. and Clin. Med.*, 1941, **26**, 1803.

[3] Karlson, K. E., Dennis, C., and Westover, D. E., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 225.

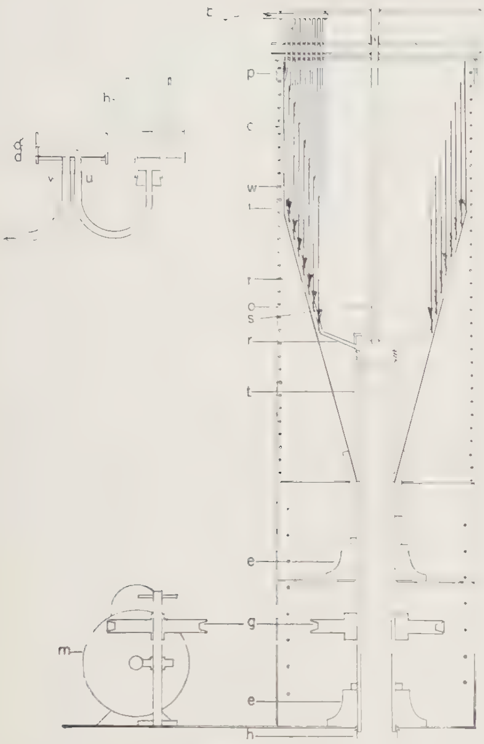

FIG. 1.

Schematic representation of oxygenator. b. blood inlet; c, cylinders upon which blood films; d. rubber dam covering outlet; d′, rubber dam raised to permit flow; e, bearing; f, blood collecting funnel; g, pulleys for revolving oxygenator; h, collecting cup; j. insulated jacket; l, level of blood when rubber dam shuts off blood flow; l′, level of blood when rubber dam is raised; m, motor; o, oxygen inlet tube; p, plastic cover; r, rack which holds concentric cylinders and conducts oxygen to the cylinders; s, spouts which deliver oxygen to each cylinder; t, hollow shaft which delivers blood to cup, h; u, blood inlet tube to level control; v, blood outlet tube from level control to pump; w, warm water coil.

6% $CO_2$ gas mixture is piped down into the shaft (o) and through the hollow tubing of the rack (r) which supports the cylinders. There are four oxygen inlets (s) supplying each cylinder. The shaft, cone, and cylinders all rotate as one unit at 150 r.p.m. There is very little foaming of blood.

The blood is pumped from the animal and back to the animal by pumps which have an intermittent pulsatile action. Ball valves give directional flow. Steady flow into the oxygenator is obtained by having 3 such pumps in a parallel arrangement.

The blood level in the collecting cup is maintained by a leveling device which consists of a plate with a sheet of rubber dam (d) stretched over it. A hole in the plate admits blood from the collecting cup, another allows the blood to flow into the "arterial" pump. The level of the plate in relation to the cup determines the level of blood in the cup because hydrostatic pressure of blood in the cup raises the rubber dam (d′) off the plate and allows blood to flow under the dam to the pump. When the level of blood in the cup is at the level of the plate (1) the rubber dam is sucked down onto the plate by the pump and no blood flows. Because the action of the pump is intermittent, the dam is again separated from the plate by the blood when the level in the cup rises (1′). Body temperature is maintained with warm water coils (w) around the oxygenator and warm air around the collecting cup and leveling device. A filter with 230 micra spaces is used in the "arterial" stream.

To date 13 perfusions of dogs have been done. In 4 instances blood was withdrawn from the femoral veins, and in 9 the vena cava was cannulated. Blood was infused via the femoral, brachiocephalic, or the subclavian artery. Blood flow rates were estimated with a venturimeter. The volume of the film was measured by turning off the pumps to the oxygenator and allowing the blood on the cylinders to collect in a graduate. Oxygen content of blood as it left the animal (venous) and as it returned (arterial) was determined by the Van Slyke method.[4]

*Results.* The results of 5 experiments in which data were collected are tabulated in Table I. At the flow rates obtained by pumping all available blood from the vena cava a film could be maintained on only 4 or 5 cylinders. Thus, the total capacity of the oxygenator is considerably greater than that utilized in these instances. It was noted that the animals made very few slight respiratory movements during perfusion even though no pulmonary respiration was maintained. The efficiency of the oxygenator (no. of cc $O_2$

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[4] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE I.
Results of Five Perfusions Withdrawing Blood from the Vena Cava and Returning It Through a Femoral Artery.

| Length of perfusion (min.) | Blood flow (cc/min.) | Film vol. (cc) | No. of cylinders filmed | $ccO_2$ introd. per cc film per min. | $ccO_2$ introd. per min. | Survival time | Plasma hemoglobin (g%) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Before | After |
| 10½ | 720 | 300 | 4 | .272 | 81.7 | 6 wk* | | |
| 12 | 680 | 270 | 5 | .272 | 73.4 | 1½ hr | .57 | .54 |
| 11 | 840 | 210 | 5 | .284 | 59.6 | 10 min. | .25 | .23 |
| 21 | 760 | 200 | 4 | .266 | 53.2 | 1½ hr | .70 | .71 |
| 22 | 1250 | 265 | 6 | .295 | 78.3 | 3 hr | .80 | .94 |

* Alive.

introduced into the blood per cc of film per minute) is seen to compare favorably with that in earlier experiments (0.247 cc). There was one other survival. The remainder expired at periods of 30 minutes to 36 hours after perfusion. Hemolysis was found to increase very little during perfusion.

*Conclusions.* 1. An oxygenator consisting of 8 concentric revolving cylinders which utilizes a funnel to deliver the blood into a small cup is described.

2. Animal perfusions demonstrate that its efficiency as an oxygenator is high enough to make it possible to perfuse the entire animal body without using an impractically large amount of blood in the extra-corporeal circulation.

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## 17135. Hemagglutination by Normal and Poliomyelitis Stool Suspensions.*

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The phenomenon of agglutination of erythrocytes by certain viruses is well known.[1] Viruses known to produce hemagglutination include influenza A & B, vaccinia, ectromelia, mumps, mouse pneumonitis, fowl plague and Newcastle disease. These agglutination reactions can be differentiated from each other by inhibition with specific immune sera. Certain bacteria and pleuropneumonia organisms also have the property of agglutinating red blood cells. Recently, the presence of a hemagglutinin in bacteria-free amniotic fluid of normal embryonated hens eggs has been described by the Army Commission on Acute Respiratory Diseases.[2] This substance was shown to be associated with the globulin fraction of egg albumin and gains access to amniotic fluid when the contents of the albumin sac ruptures into the amniotic cavity between the 11th and 13th day of incubation. Hemagglutination can also be induced by ricin, abrin, crotin and certain inorganic colloidal acids or bases such as salicylic acid and basic proteins as protamine.

In studies on the detection of possible hemagglutination by the poliomyelitis virus, it was found that both normal and infected saline stool suspensions agglutinated the erythrocytes of a wide variety of animal species. This paper is a report describing the agglutination of group "O" human, chick, rabbit, guinea pig and sheep erythrocytes by stool suspensions and some of the properties of the stool hemagglutinin.

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* Supported in part by the Michael Reese Research Foundation.

[1] Rivers, T. M., Viral and Rickettsial Infections of Man, J. B. Lippincott Co., 1948, p. 77.

[2] Commission on Acute Respiratory Diseases, PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 118.

TABLE I.
Hemagglutination of Different Species by Normal and Poliomyelitis Stool Suspensions.

| Stool No. | Final agglutination titer<br>Human group O | Chick | Rabbit | Guinea pig | Sheep |
|---|---|---|---|---|---|
| | | Poliomyelitis stools. | | | |
| 7 | 1:30 | 1:30 | 1:20 | 1:20 | 1:20 |
| 16 | 1:20 | 1:40 | 1:160+ | 1:20 | 1:20 |
| 17 | 1:320+ | 1:320+ | 1:320+ | 1:60 | 1:320+ |
| 18 | 1:120 | 1:40 | 1:320+ | [illegible] | [illegible] |
| | | Normal stools. | | | |
| 3 | 1:20 | 1:30 | 1:320+ | 1:40 | 1:20 |
| 4 | 1:40 | 1:40 | 1:160 | 1:20 | 1:20 |
| 5 | 1:20 | 1:20 | 1:320+ | 1:20 | 1:20 |
| 6 | 1:120 | 1:120 | 1:320+ | 1:40 | 1:80 |

TABLE II.
Inhibition of Stool Hemagglutination by Serum and Bovine Albumin Using Chick Cells.

| Exp. | Serum | Serum dilution<br>1:6 | 1:12 | 1:24 | 1:48 | 1:96 | 1:192 | 1:384 | 1:768 | Titer |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Human | 0 | 0 | 0 | 0 | ± | + | + | + | 1:48+ |
| 2 | Rabbit | 0 | 0 | 0 | 0 | + | + | + | + | 1:48 |
| 3 | Monkey | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ± | 1:384+ |
| 4 | 20% bovine albumin | 0 | 0 | 0 | + | + | — | — | — | 1:24 |

0 = No agglutination.
+ = Agglutination.

*Methods.* 10% stool suspensions were prepared by grinding the stools in a mortar and pestle with a small amount of physiological saline, diluting the suspensions to a final concentration of 10% and centrifuging in an angle centrifuge at 5000 r.p.m. for 20 minutes. The supernatant was then removed and cultured for the presence of bacteria. Titrations were carried out by making serial 2-fold dilutions of stool suspension in saline. 0.5 cc of material was added to each test tube. Next equal parts of 0.75% of the various erythrocyte suspensions which had been previously washed 3 times prior to use were added. The tubes were mixed and allowed to stand at room temperature for 1 hour prior to reading. The titer was read as the highest dilution showing a hemagglutination pattern on the bottom of the test tube. This pattern closely resembled that produced by influenza and other viruses.

*Results.* Titrations obtained comparing poliomyelitis and normal stool suspensions with group "O" human, chick, rabbit, guinea pig and sheep erythrocytes are shown in Table I. Highest titers were obtained with rabbit erythrocytes while titers with the erythrocytes of other species did not differ significantly. It was found that rabbit cells in saline occasionally showed spontaneous agglutination and such experiments were discarded. The results summarized in Table I indicate that there was no significant difference in hemagglutination titers between normal and poliomyelitis stool suspensions. In other experiments it was found that a 10% mouse brain and cord suspension infected with the Lansing strain of poliomyelitis virus failed to agglutinate chick, human group "O" and rabbit cells.

Table II shows the inhibition of hemagglutination of chick erythrocytes by human, rabbit, and monkey serum and 20% bovine albumin in relatively high dilutions. Salk's technic[3] of the hemagglutination-inhibition test for antibodies against the influenza virus was employed. Similar results were obtained in hemagglutination-inhibition tests with the erythrocytes of other species. The effect of heat on hemagglutination of chick cells by

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[3] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE III.
Effect of Heat on Hemagglutination by Stool Suspensions of Chick Cells.

| Stool No. | Temperature °C | Time | Titer |
|---|---|---|---|
| 3 | Unheated | — | 1:30 |
| | 65 | 15 min. | 0 |
| 7 | Unheated | — | 1:400 |
| | 56 | 30 min. | 1:60 |
| | 65 | 15 min. | 0 |
| 17 | Unheated | — | 1:120 |
| | 65 | 15 min. | 0 |
| 18 | Unheated | — | 1:40 |
| | 65 | 15 min. | 0 |

TABLE IV.
Hemagglutination of a Stool Suspension after Previous Absorption with Human Group O, Chick and Rabbit Erythrocytes.

| | Final agglutination titer | | | |
|---|---|---|---|---|
| Absorbing cells | Human group O | Chick | Guinea pig | Rabbit |
| Group O | 1:10 | 1:20 | 1:20 | 1:240? |
| Rabbit cells | 1:60 | 1:20 | 1:20 | 1:20 |
| Chick cells | 1:20 | 1:20 | — | — |
| None | 1:240 | 1:240 | 1:60 | 1:320+ |

stool suspensions is shown in Table III. Incubation in a water bath at 65°C for 15 minutes completely destroyed the stool hemagglutinin in the experiments listed in Table III, while heating at 56°C for 30 minutes also reduced the titer significantly. In other experiments, the hemagglutination titer was approximately the same when carried out at 3-5°C, room temperature and 37°C. Storage for 2 weeks in the icebox resulted in a marked drop in titer. Stool suspension Seitz filtrates failed to show hemagglutination, but titers could be partially restored (¼ to ½) the original) by methanol concentration at low temperatures.[4]

The hemagglutinin could be absorbed in differential erythrocyte absorption experiments by group "O" human, chick and rabbit erythrocytes (Table IV). The absorption was carried out by adding equal parts of 50% packed cells to the stool suspension, incubating for 15 minutes at room temperature and centrifuging at 1500 r.p.m. for 15 minutes. The supernatant was then drawn off and tested for the presence of hemagglutinins against homologous and heterologous erythrocytes. Usually a single absorption was adequate for almost complete removal of the hemagglutinins.

*Discussion.* The relationship of the stool hemagglutinins to those produced by viruses, bacteria and normal amniotic fluid is unknown. The stool hemagglutinins resemble those produced by bacteria more closely because both are thermolabile, decrease in titer on storage and are inhibited by normal serum. On the other hand, the virus hemagglutinins are more heat stable and are inhibited only by specific sera.

Occasionally the stool suspensions were bacteriologically sterile but generally *Escherichia coli, Staphylococcus,* yeasts or diphtheroids were isolated. Turbid saline suspensions of the various isolated organisms (containing approximately 1 billion per cc) were tested for agglutination with chick cells, group "O" human, rabbit and guinea pig cells. No hemagglutination was observed with any of the cells tested except the rabbit erythrocytes which agglutinated in relatively low titers (1:2 to 1:6 dilutions of the bacterial suspensions). These results are in accord with those recently reported by Griffitts.[5] This worker found that *E. coli, Aerobacter aerogenes, Alcaligenes faecalis, Bacillus anthracis, Brucella abortus, Corynebacterium diphtheriae, Eberthella typhosa, Proteus vulgaris, Salmonella, Shigella, Staphylococcus aureus* and a few other bacterial species failed to agglutinate human erythrocytes. On the other hand, 23 strains of *Shigella alkalescens*, some strains of *Hemophilus pertussis, Vibrio comma*, and *Streptococcus pyogenes* showed hemagglutination of human erythrocytes. None of the species of bacteria which Griffitts reported to produce hemagglutination were isolated from stool suspensions cultured in the present study.

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[4] Cox, H. R., *et al.*, *J. Immunol.*, 1947, **56**, 149.

*Summary.* Saline suspensions of stools obtained from both normal and poliomyelitis patients showed hemagglutination of group "O" human, chick, rabbit, guinea pig and sheep erythrocytes. The rabbit cells were agglutinated in highest titers. No significant difference in hemagglutination titer was noted with the normal and poliomyelitis stools. Normal human, rabbit and monkey serum and 20% bovine albumin inhibited hemagglutination. The hemagglutinin was thermolabile and absorbed by group "O" human, chick and rabbit erythrocytes. Stool suspension Seitz filtrates failed to show hemagglutination, but the titer could be partially restored by methanol precipitation. Various species of bacteria isolated from stool suspensions failed to agglutinate chick, group "O" human and guinea pig erythrocytes but showed some agglutination of rabbit cells in low titer. Mouse brain and cord suspensions infected with the Lansing strain of poliomyelitis virus also failed to agglutinate chick, human group "O" and rabbit erythrocytes.

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[5] Griffitts, J. J., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 358.

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## 17136. An Antithyrotoxic Factor for the Rat Not Identical with Vitamin $B_{12}$.*

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It has been demonstrated that whole liver powder will prolong survival and counteract growth retardation of immature rats fed massive doses of desiccated thyroid.[1-3] Some confusion exists, however, regarding the nature of the factor or factors responsible for the above effects. On rations containing soybean meal the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein has been counteracted both in the rat and chick by liver fractions with vitamin $B_{12}$ activity.[4-6] In subsequent work similar findings were obtained with crystalline vitamin

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Communication No. 203 from the Department of Biochemistry and Nutrition, University of Southern California.

[1] Ershoff, B. H., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 500.

[2] Ershoff, B. H., *Arch. Biochem.*, 1947, **15**, 365.

[3] Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

[4] Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, J. W., and Barnes, R. H., *J. Nutrition*, 1949, **37**, 21.

[5] Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

[6] Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 631.

$B_{12}$.[7,8] On rations containing casein as the dietary protein and sucrose as the dietary carbohydrate, however, the retardation in growth following administration of massive doses of thyroactive substances appears to be due to a deficiency of some other factor. This latter substance which has been termed the "antithyrotoxic factor of liver" is present in considerable concentration in the water-insoluble fraction of liver.[2,9] It is not present in significant amounts in liver fractions with vitamin $B_{12}$ activity or other concentrates of vitamin $B_{12}$.[10] In the present communication further data are presented concerning the ineffectiveness of crystalline vitamin $B_{12}$ as a source of antithyrotoxic factor for the immature hyperthyroid rat.

*Procedure and results.* The basal ration employed in the present experiment consisted of sucrose, 73.0%, casein,[†] 22.0%, salt mixture,[‡] 4.5%; and U.S.P. desiccated thyroid,[§] 0.5%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg, pyridoxine hydrochloride, 15 mg, calcium pantothenate, 67.2 mg, nicotinic acid, 60 mg, 2-methyl-naphthoquinone, 5 mg and choline chloride 1.2 g.[‖] Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg and a vitamin A-D concentrate containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.[¶] In addition to the basal ration the following diets were also employed, consisting of basal ration plus each of the following supplements: (1) 30 $\gamma$ of vitamin $B_{12}$ per kg of diet,[**] (2) 10% whole liver powder,[††] (3) 10% extracted liver residue,[‡‡] and (4) 4% liver concentrate 1-20.[§§] The liver fractions were added in place of an equal amount of sucrose. In addition to the above, two control diets were also tested. These consisted of (1) basal ration with thyroid omitted and (2) a similar ration supplemented with 30 $\gamma$ of vitamin $B_{12}$ per kg of diet. Sucrose replaced thyroid in both of the latter rations. Sixty-two female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 46.0 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* Feeding was continued for 28 days or until death, whichever occurred sooner. Results are summarized in Table I.

In agreement with earlier findings whole liver powder completely counteracted the growth retardation of immature rats fed massive doses of thyroid. The protective factor was retained in the water-insoluble extracted liver residue. Little if any activity was present in the water-soluble liver concentrate 1-20. Crystalline vitamin $B_{12}$ was inactive. These findings indicate that extracted liver residue

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[7] Nichol, C. A., Dietrich, L. S., Cravens, W. W., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 40.

[8] Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 392.

[9] Ershoff, B. H., and McWilliams, H. B., *Science*, 1948, **108**, 632.

[10] Ershoff, B. H., *Exp. Med. and Surg.*, 1948, **6**, 438.

[†] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[‡] Sure's Salt Mixture No. 1.[11]

[11] Sure, B. J., *J. Nutrition*, 1941, **22**, 499.

[§] Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

[‖] In view of the increased requirements for thiamine, pyridoxine and pantothenic acid in the hyperthyroid rat,[12] the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

[12] Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1942, **135**, 474.

[¶] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

[**] The vitamin $B_{12}$ employed in the present experiment was obtained from Merck and Co., Rahway, N. J.

[††] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

[‡‡] Extracted Liver Residue, Wilson Laboratories, Chicago, Ill. This material consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

[§§] Liver Concentrate Powder 1-20, Wilson Laboratories, Chicago, Ill. This material consists of the water-extractable material of raw liver.

TABLE I.
Comparative Effects of Vitamin $B_{12}$ and Various Liver Fractions on the Growth of Immature Rats Fed Massive Doses of Desiccated Thyroid.

| Supplements fed with basal ration | % thyroid | No. of animals | Initial body wt, g | Avg gain in body wt after 28 days of feeding,* g |
|---|---|---|---|---|
| None | .5 | 10 | 46.1 | 47.8 ± 4.0<br>(6) |
| Whole liver powder | .5 | 10 | 46.0 | 109.7 ± 2.1<br>(8) |
| Extracted liver residue | .5 | 10 | 46.0 | 103.4 ± 4.4<br>(9) |
| Liver concentrate 1-20 | .5 | 10 | 46.1 | 63.3 ± 3.2<br>(9) |
| Vit. $B_{12}$ | .5 | 10 | 46.1 | 48.3 ± 3.5<br>(7) |
| None | .0 | 6 | 45.7 | 98.8 ± 5.8<br>(6) |
| Vit. $B_{12}$ | .0 | 6 | 45.8 | 97.6 ± 3.9<br>(6) |

The values in parentheses indicate the number of animals that survived of which this is an average.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

contains one or more factors other than vitamin $B_{12}$ which were responsible for its antithyrotoxic effects under conditions of the present experiment.

Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal.[13] This is particularly true for some of the B vitamins. An increased requirement for thiamine,[12,13] pyridoxine,[12,13] pantothenic acid,[12,13] folic acid[14] and more recently vitamin $B_{12}$[7,8] has been demonstrated following administration of large doses of thyroactive substances. In addition to the above, requirements are increased for at least one additional factor in the hyperthyroid animal. This substance, which has been termed the "antithyrotoxic factor of liver", is retained in the water-insoluble fraction of whole liver powder. Recent findings[2,3] with those of the present experiment indicate that this factor is distinct from any of the known nutrients including vitamin $B_{12}$.[|||]

*Summary.* Growth was markedly reduced in hyperthyroid rats fed purified rations containing casein as the dietary protein and sucrose as the dietary carbohydrate. The retardation in growth was completely counteracted by the administration of a water-insoluble fraction of liver. Crystalline vitamin $B_{12}$ was ineffective. The protective factor in liver is distinct from any of the known nutrients including vitamin $B_{12}$.

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[13] Drill, V. A., *Physiol. Rev.*, 1943, **23**, 355.

[14] Martin, G. J., *Amer. J. Dig. Dis.*, 1947, **14**, 341.

[|||] While this paper was in press Betheil and Lardy[15] reported that crystalline vitamin $B_{12}$ *partially* counteracted the growth retardation of immature male rats fed massive doses of thyroid in a ration similar to that employed in the present experiment. Growth was significantly less, however, than that occurring in animals fed a similar diet containing 10% whole liver powder. It would appear that Betheil and Lardy were dealing with a multiple deficiency induced by thyroid feeding. Vitamin $B_{12}$ was one of the deficient factors, but optimum growth was not obtained unless animals were supplied an additional factor or factors present in whole liver. Emerson[16] has recently found that Vitamin $B_{12}$ may be stored in considerable amounts in the tissues of the young rat during the period of pregnancy and lactation. It is not unlikely that differences in the growth of immature hyperthyroid rats fed vitamin $B_{12}$ may be due, at least in part, to the vitamin $B_{12}$ content of the pre-test dietary regime.

[15] Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, **37**, 495.

[16] Emerson, G. A., *Fed. Proc.*, 1949, **8**, 381.

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# 17137. Increased Portal Pressure and Ascites in Rats Following Ligation of Portal Vein.

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Numerous reports have appeared in the literature[1-3] concerning the failure to produce ascites in animals by ligation of the portal vein. These results have caused some people to minimize the importance of increased portal pressure as a factor in the mechanism of ascites in patients with cirrhosis of the liver. Recently Blakemore[4] and Linton[5] have demonstrated that ascites sometimes disappears following anastomoses between the portal and the general venous circulation. Portal pressures 3 and 4 times the nomal range have been found at operation in patients with cirrhosis accompanied by ascites. However, the exact role of elevated portal pressure in the production of ascites is not clear.

The present experiments were undertaken to assess the influence of hypoproteinemia, increased Na intake and antidiuretic substances on the production of ascites in rats with elevated portal pressure. It was found that partial ligation of the portal vein alone produced some ascites in a large percentage of the rats. These animals were demonstrated to have increased portal pressure. The additive effect of the other procedures on ascites will be described in another communication.

*Experimental Procedure and Results.* Adult rats weighing 200-300 g were operated on under ether anesthesia and the portal vein above the splenic vein was ligated to approximately ¼ its original circumference. This was carried out by exteriorizing the portal vein and tying it off completely. Then a small probe was inserted in the loose knot and it was dilated to the desired circumference. If the ligation was complete, all animals died within a few hours. The portal pressure in such animals reached values as high as 1,000 mm $H_2O$ and numerous small vessels were ruptured. In addition, there was marked engorgement of blood, giving a dark red color to the intestines. In the partial ligation an effort was made to allow just enough portal blood to pass to prevent the death of the animal. The color of the intestines helped to determine the degree of obstruction. It was possible to raise the portal pressure as much as 200 mm of water by the partial ligation and still obtain 85% survival. Complete ligation of the portal vein was carried out in 2 stages in 42 rats by the method of Whitaker.[6] This was a somewhat cumbersome procedure and did not give significantly higher portal pressures than the one-stage partial ligation. A 60% survival rate was obtained for the 2-stage complete ligation.

Following partial ligation of the portal vein, ascites was carefully sought both by laparotomies and by placing a needle in the abdomen. Ascites was found in 69% of the animals. Table I shows the incidence of ascites in 110 rats which received the partial ligation as compared to a control group which received only intestinal manipulation at the time of operation. The fluid obtained resembled human ascitic fluid with a slightly higher protein content (an average of 2.2%). The ascites was found to develop usually on the second day after the operation, reach a peak at approximately the 6th day and then gradually disappear so that few of the rats had any ascites after the 14th day. The ascites could easily be missed and was often not evident from the weight changes of the animals. Massive ascites requiring removal of fluid from the

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[1] Eck, N. V., *Voyena Med. J.*, 1877, **130**, 1.

[2] Bollman, J. L., *Trans. Liver Injury Conference*, Josiah Macy, Jr. Foundation, 1948, **7**, 21.

[3] Milnes, R. F., and Child, C. G., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 332.

[4] Blakemore, A. H., *New York State J. Med.*, 1947, **47**, 479.

[5] Linton, R. R., Hardy, I. B., and Volwiler, W., *Surg., Gynec. and Obst.*, 1948, **87**, 129.

[6] Whitaker, W. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 420.

TABLE I.
Comparison of the Amount of Ascites Formed in a Group of Rats with Partial Ligation of the Portal Vein and in an Operated Control Group.

| Operation | No. of rats | Rats with ascites | | | |
|---|---|---|---|---|---|
| | | 1-4 cc | | >4 cc | |
| | | No. | % | No. | % |
| Intestinal manipulation | 26 | 0 | 0 | 0 | 0 |
| Portal vein ligation | 110 | 60 | 55 | 16 | 14 |

abdomen developed in only 3 of the rats. The 2-stage complete ligation of the portal vein did not cause a higher incidence of ascites.

In view of the fact that the occurrence of ascites appeared to be quite unpredictable in the rats with partial ligation of the portal vein, the pressure in the portal circuit was determined. This was carried out by means of a simple small diameter manometer that was connected to a needle which was inserted into branches of the mesenteric veins. Heparinized saline was used in the manometer and the results obtained were corrected for capillarity. Anaesthesia was found to increase considerably the pressure in the portal system and the pressure could be made to rise and fall according to the degree of anesthesia. It was possible, however, to bring all the animals to a constant stage of anaesthesia so that the pressures could be compared. Normal animals gave pressures ranging from 100-160 mm $H_2O$. All animals with partial ligation showed values which were higher than the highest normal. Table II shows the portal pressure in 9 rats along with the amount of ascites. It is evident that there is no direct correlation. However, there was a general tendency for the animals with the higher pressures to have more ascites. The cause of the large amount of ascites in some of the animals was not clear. It appeared as though other factors were operating in these animals in addition to increased pressure. With time the portal pressure gradually declined and usually fell to a level just above normal at the end of 3 weeks.

TABLE II.
Comparison of the Portal Pressure and the Volume of Ascitic Fluid in 9 Rats Operated on 6 Days After Partial Ligation of the Portal Vein.

| Rat No. | Portal pressure, mm $H_2O$ (Normal 100-160) | Ascites, cc |
|---|---|---|
| 1 | 350 | 4 |
| 2 | 320 | 2 |
| 3 | 310 | 3 |
| 4 | 280 | 0 |
| 5 | 270 | 10 |
| 6 | 250 | 0 |
| 7 | 230 | 2 |
| 8 | 220 | 0 |
| 9 | 210 | 0 |

*Summary*. Partial ligation of the portal vein resulted in the development of a small amount of ascites in 69% of a group of 110 rats. All rats showed an increase in portal pressure but the degree of increase did not correlate well with the amount of ascites.

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# 17138. Dosage-Mortality in Rats Given Total Body Roentgen Irradiation.*

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In a previous communication[1] we indicated that certain flavonoid compounds may decrease the mortality of guinea pigs given approximately an $LD_{50}$ of total body roentgen radiation.

In attempts to extend these and other treatments to other species of small laboratory animals, some results have been obtained on dosage-mortality in the rat, which may be of value to others working on the biological effects of ionizing radiation.

We have been unable to find previous published mortality data for the rat other than a note by Potter,[2] who reported 100% death in 14 rats with total body radiation of 800 r. Prosser, *et al.*[3] state that the 30-day M.L.D. for single doses of x-radiation is 600 r† for rats but no particulars were given.

In our studies on the rat, over 500 animals have been used. The purpose of this communication is to report the dosage-mortality data for normal rats.

The rats used were of the Slonaker albino stock, from which the Wistar albino stock originated and which have been inbred in this laboratory since 1929.[4] Their food consisted of Purina laboratory chow available on the meshscreen cage floors and tap water from bottles with glass spouts. In all cases the animals were healthy young males, average body weight 100-140 g.‡ Six animals were caged together. The room temperature was 78° ± 2°F.

The animals to be irradiated were placed in a ventilated 6-partitioned plywood box, 6 at a time. The dimensions of each compartment were 2″ wide, 5″ long (adjustable) and 3″ deep. The partitions were ⅛″ plywood and the top was ¼″ plywood perforated with holes. The bottoms were ¼″ hardware screen and beneath this was a layer of rice 3 inches in depth which was used to provide additional backscatter.§

The X-ray factors were: GE Model KX-3, 220-kv deep therapy unit, 200 kv, 20 MA, ½ mm Cu and 1 mm Al added filtration (HVL, 1.05 mm Cu) 52.5 cm target distance to top of box, 30.4 r/min. in air single exposure total body irradiation. The times were varied to give the desired dosage. The unit is calibrated semi-annually by a registered X-ray physicist. The variation in output over the past year has been a little under 3%. This of course does not ensure accurate control from day to day, but since control animals always were under the beam at the same time as the experimentals, this objection is not so serious.

It was found that if rats survived longer than 30 days they generally survived indefinitely. Hence, 30 days were taken as a

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[1] Clark, W. G., Uncapher, R. P., and Jordan, M. L., *Science*, 1948, **108**, 269.

[2] Potter, J. C., *Radiology*, 1941, **37**, 724.

[3] Prosser, C. L., Painter, E. S., Lisco, H., Brues, A., Jacobson, L. O., and Swift, M. N., *Radiology*, 1947, **49**, 299.

† A. H. Dowdy (personal communication) informed us that the Rochester Unit of the Manhattan District found an $LD_{50}$ of 650 r for Wistar rats, which closely agrees with the results reported below.

[4] MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927-8, **83**, 179.

‡ A wider weight distribution should not be used, since Naiman[5] has shown a considerable effect of age on susceptibility of rats to total body x-radiation.

[5] Naiman, D. N., *Am. J. Roentgenol.*, 1949, **61**, 95.

§ Ellinger[6] has shown in mice that additional backscatter of this nature reduces the steepness of the slope of the sigmoid mortality curve in the $LD_{50}$ range.

[6] Ellinger, F., *Radiology*, 1945, **44**, 125.

TABLE I.
X-ray Dosage-Mortality in Rats.

| r. | No. rats | 50% dead (days) | % mortality (30 days) |
|---|---|---|---|
| 1000 | 8 | 6 | 100 |
| 800 | 32 | 8 | 90 |
| 750 | 8 | 8 | 100 |
| 715 | 8 | 8 | 100 |
| 670 | 8 | 16 | 53 |
| 655 | 235 | 15 | 79 |
| 640 | 83 | 30 | 41 |
| 625 | 94 | 45 | 62 |
| 610 | 8 | | 0 |
| 500 | 17 | | 6 |
| | 501 | | |

convenient endpoint for expressing the data, although in most instances observations were continued for 45-60 days or longer. For all practical purposes the present mortality data include indefinite survival.

Table I lists the results on 501 rats.

The dosages (column 1) were plotted against per cent mortality (column 4) as shown in Fig. 1.

Fig. 2 relates the survival time to per cent mortality, for each dosage. Because of overlapping, it was found most useful to pool the data as indicated.

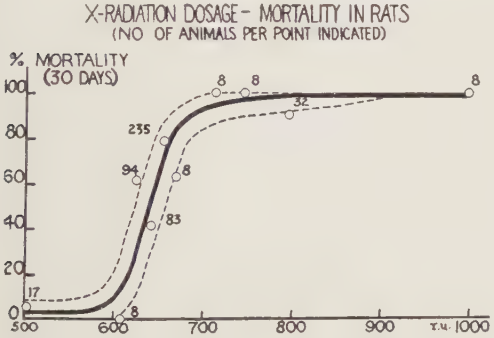

FIG. 1.

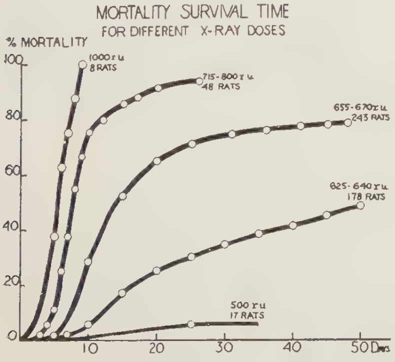

FIG. 2.

The time in days lapsing before 50% of the animals died was plotted against dosage, in Fig. 3. This relationship has been found useful in assessing the effects of drugs on radiation disease.[1]

When the data were plotted by Miller and Tainter's modification[7] of Litchfield and Fertig's method,[8] on logarithmic-probit graph paper, the $LD_{50}$ was approximately 640 ± 5 r. However, it was difficult to fit the points to a satisfactory straight line by this method because of the variations in the steep part of the curve, and from Fig. 1 it is evident that from 610 to 655 r (± 3%), a 0 - 86% mortality may occur. The ± 3% is the calibrated limit of variation of the X-ray machine, so that any results from 0 to nearly 100% mortality could be expected within the "$LD_{50}$" range because of the steepness of the slope in this range. Only unless very large numbers of animals were used together with radiation dose variation in the hundredth per cent range, could this portion of the curve be duplicated from one experiment to the next.

Table II lists some calculations from our data on the rat, compared with those of Ellinger[6,9] for goldfish, guinea pigs and mice, and of Tullis, *et al.*[10] for swine; of the rate

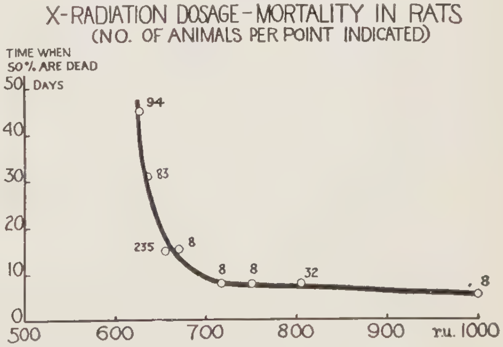

FIG. 3.

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[7] Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261.

[8] Litchfield, J. T., Jr., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 276.

[9] Ellinger, F., *Radiology*, 1940, **35**, 563.

[10] Tullis, J. L., Tessmer, C. F., Cronkite, E. P., and Chambers, F. W., *Radiology*, 1949, **52**, 396.

TABLE II.
Mortality Rate per r. Unit—Species Differences.

| Species | No. animals used | dx/dy* | Reference |
|---|---|---|---|
| Rats | 500 | 1.2% | Clark and Uncapher |
| Guinea pigs | 50 | 0.7 | Ellinger[6] |
| Swine | 32 | 0.5 | Tullis *et al.*[10] |
| Mice | 119 | 0.3 | Ellinger[6] |
| Goldfish | 774 | 0.08 | Ellinger[9] |

* $x$ = % mortality and $y$ = dosage in r, in the $LD_{50}$ range.

of mortality per roentgen unit dose, in the steepest part of the mortality curves. This is an expression of the slope, $dx/dy$, where $x$ represents unit per cent mortality and $y$ the unit dose.

This means that 1.2% of the rats died per unit increment in dosage of X-rays, where the other species died at a lower rate per dosage increment.

It will be interesting to compare these data with those for the dog, rabbit, monkey, cat and other species.

It occurs to us that in studies directed toward the therapy or protection by pre-treatment of animals given total body ionizing radiation, that if the mortality curves are quite steep, as in the rat, dosages in the "$LD_{50}$" range may not be permissible, and that dosages should be given which consistently kill 100% of the untreated animals, so that an estimation of protection could be made by both the time required to kill 50% of each group, as shown in Fig. 3, and by the per cent final mortality. If the slope is not so steep ($dx/dy$ is small) and if sufficient animals are used, it would be advisable to include at least 3 dosages, $LD_{100}$, $LD_{50-60}$ and $LD_{20-30}$.

*Summary.* The $LD_{50}$ for total body X-radiation in Wistar rats is 640 ± 5 r with a 30-day endpoint. The time elapsing before 50% mortality occurs at different doses is a more reliable measure of mortality than the dosage-mortality curve or the dosage-survival time curves for different X-ray doses. The dosage-mortality curve of rats has such a steep slope in the $LD_{50}$ range that variations in mortality of from 0 to nearly 100% may occur in this range because of variations in animals and in the X-ray machine output. Therefore in studies directed toward therapy or protection of animals given total body ionizing radiation, if the mortality curves are extremely steep as in the rat, dosages in the $LD_{50}$ range should not be used. Other measures which might be employed are discussed.

We wish to express our gratitude to Dr. F. Ellinger, Director of Radiological Research, Naval Medical Research Institute; to Dr. A. H. Dowdy, chairman, Department of Radiology, School of Medicine, University of Calif.; and to Dr. V. P. Bond and Peggy Smith of the Navy Radiological Defense Laboratory, San Francisco Naval Shipyard, for reading this manuscript and making certain suggestions.

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## 17139. Resemblance of a Strain of Swine Influenza Virus to Human A-prime Strains.*

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*From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich.*

In the course of studies in a group of children vaccinated with the Type A PR8 strain of influenza virus, an outbreak of influenza occurred in that population.[1] The children's sera were being tested against different strains of influenza virus, and it was observed that the serological reactions to a strain of virus of swine origin were similar to those obtained with strains isolated during the 1947 outbreak in the civilian population of the United States.[2-4] The evidence strongly suggests that this swine strain (Oti) is closely related to strains of human origin more recently studied which are being called A-prime.

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* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiology Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[1] Quilligan, J. J., Jr., Minuse, Elva, and Francis, Thomas, Jr., *J. Clin. Invest.*, 1949, **27**, 572.

*Materials and methods. Description of Virus Strains.* The virus preparations used in the agglutination inhibition tests were taken from infected allantoic fluid. The passage history of each strain is as follows: Type A RP8[5]—7 ferret, 593 mouse and 69 egg passages; A-prime Rhodes[2]—4 ferret, 7 mouse, and 13 egg passages; A-prime FMl[3] (obtained from Dr. J. E. Smadel)—37 mouse and 15 egg passages; Swine 1976 (obtained from Dr. R. E. Shope)—38 mouse and 19 egg passages; Swine Oti, a strain sent to Dr. R. E. Shope by Dr. M. Tsurumi of the Imperial University of Nagoya, Japan. He, in turn, had received it from Dr. Oti, who originally isolated it from swine in Korea in 1939. Dr. Shope noted that this strain was different in its behavior from the American swine strains, and after being received in this laboratory in 1944 after 179 mouse passages it was soon realized that it acted a great deal like a type A human strain. Since then it has been passed 33 times in eggs.

*Sera from Children.* There were 175 children in the institution where the vaccination study was carried out; 56 recognized cases of influenza appeared among them between late February, 1947 and the middle of April, 1947. Serum had been obtained from most of the children at the termination of the vaccination study, in early November 1946, four months before the outbreak of influenza. Beginning one week after the last clinically recognized case, approximately seven months after the last previous sample, another specimen of serum was obtained from 52 children, eleven of whom had been noticeably ill during the epidemic period.

*Sera from Adults.* A group of 93 pairs of acute and convalescent sera were obtained from patients admitted to the student Health Service Infirmary of the University of Michigan during the period of the outbreak.[2] Fifty-eight of them had been inoculated with a vaccine of combined A and B influenza viruses.

*Sera from Animals.* All animal sera, with one exception, were from ferrets which received intranasal inoculations of the allantoic fluids containing the specific viruses. Sera were generally obtained before and 2 weeks after inoculation. Strict isolation technic was used and only single strains of virus were introduced into the ferret isolation unit to eliminate the possibility of cross-infection of the animals. One anti-serum prepared in rabbits (inoculated with the Oti strain in 1944) was used to check the identity of the current Oti line that was used in the tests. The results indicated that the current Oti line reacted the same as it had before any A-prime strains had been isolated in this laboratory.

*Serological Tests.* Antibody was measured by inhibition of agglutination of chicken erythrocytes. Four agglutinating units of virus were mixed with the serially diluted sera. A pattern method[6] of interpreting the end-point was used. The titers are expressed as reciprocals of the modified means.[7]

Neutralizing antibody was measured by inoculating mice with serially diluted serum plus 500-1,000 MLD of the virus strains. The 50 per cent endpoints[8] were calculated on the basis of the lethal effect of virus during a ten-day observation period. Titers are recorded as final dilutions of serum.

*Results.* An extensive examination was made of the sera obtained from the children

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[2] Francis, Thomas, Jr., Salk, Jonas E., and Quilligan, J. J., Jr., *Am. J. Pub. Health*, 1947, **37**, 1013.

[3] Sigel, M. M., Shaffer, F. W., Kirber, M. W., Light, A. B., and Henle, W., *J.A.M.A.*, 1948, **136**, 437.

[4] Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

[5] Francis, T., Jr., *Science*, 1934, **80**, 457.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

[7] Quilligan, J. J., Jr., and Francis, Thomas, Jr., *J. Clin. Invest.*, 1947, **26**, 1079.

[8] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Effect of A-prime Influenza Virus Infection on Serological Response of the Children Expressed as the Modified Mean Antibody Titers.

| | | | Modified mean titers | | | |
|---|---|---|---|---|---|---|
| Sera from | Stage | No. | PR8 | Rho | Oti | Swine 1976 |
| Children | Pre-infection | 52 | 716 | 43 | 38 | 24 |
| | Post-infection | 52 | 625 | 160 | 136 | 35 |

TABLE II.
Comparison of Antibody Titers of Vaccinated and Unvaccinated Members of the University Group.

| | Virus strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PR8 | | Rhodes | | Oti | | Swine 1976 | |
| | Vac. | Unvac. | Vac. | Unvac. | Vac. | Unvac. | Vac. | Unvac. |
| No. of sera tested | 57 | 35 | 56 | 35 | 58 | 35 | 57 | 35 |
| Acute* | 371 | 77 | 40 | 34 | 141 | 63 | 50 | 35 |
| Conv.* | 722 | 230 | 114 | 106 | 415 | 242 | 106 | 90 |
| Fold—inc. in means | 1.9× | 3× | 2.8× | 3× | 2.9× | 3.8× | 2.1× | 2.6× |

* Modified mean titer.

TABLE III.
Effect of A-prime Influenza Virus Infection on Serological Response of Two Groups of Individuals Expressed as the % Showing Incremental Changes in Titer.

| Group | Virus strain | Decrease or no change | 2-fold rise | 4-fold or greater rise | Total No. |
|---|---|---|---|---|---|
| University students | PR8 | 30 | 33 | 37 | 92 |
| | Rho | 21 | 29 | 50 | 93 |
| | Oti | 22 | 26 | 52 | 93 |
| | Swine 1976 | 29 | 41 | 30 | 91 |
| Children | PR8 | 73 | 11 | 16 | 37 |
| | Rho | 18 | 22 | 61 | 51 |
| | Oti | 22 | 15 | 63 | 51 |
| | Swine 1976 | 53 | 29 | 18 | 51 |

following repeated inoculations of the univalent vaccine.[1] It was found that but a minimal rise in antibody to the 1976 strain of swine virus occurred and the response as measured with the Oti strain was small. However, when the sera taken after the outbreak of influenza were compared with those obtained earlier a sharp increase in titer to the Oti strain was noted (Table I). There was no significant change in the low titer against the Swine 1976 strain, nor in the high post-vaccination titer against the PR8 strain. The results with the Oti strain were essentially parallel to those measured by the Rhodes strain (A-prime) isolated during the 1947 epidemic. Since all the children in the group were vaccinated with PR8 the lack of further response to that strain could be readily ascribed to the vaccination.

The group of University students who had been sick comprised both vaccinated and unvaccinated persons. The highest convalescent mean titer and the largest incremental increase in mean titer among the unvaccinated were observed against the Oti strain but threefold rises also occurred against both the PR8 and the 1947 Rhodes strain (Table II). A definite rise to Swine 1976 strain also took place. In the vaccinated group, there was also an increase in titer against all strains used, although the incremental rises were greatest against the Oti and Rhodes strains.

TABLE IV.
Cross Hemagglutination-Inhibition and Mouse Neutralization Tests with Allantoic Fluid Antigens and Specific Ferret Antisera.

| Ferret sera | Serum stage | Virus strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PR8<br>F7M593E69 | | Rhodes<br>F4M7E23 | | FM1<br>M37E15 | | Oti<br>M179E33 | | Swine 1976<br>M38E19 | |
| | | HI | MN | HI | MN | HI | MN | HI | MN | HI | MN |
| G28 | N | <32 | <4 | <32 | — | — | — | <32 | — | <32 | — |
| PR8-F105 | I | 4096 | 1024 | <32 | 24 | — | — | <32 | <4 | <32 | <4 |
| G12 | N | <32 | — | 32 | — | <32 | — | <32 | — | <32 | — |
| PR8-F105 | I | 1024 | — | 32 | — | <32 | — | <32 | — | <32 | — |
| K24 | N | <32 | — | <32 | <4 | — | — | <32 | — | <32 | — |
| Rho-F4M7E12 | I | <32 | 4 | 2048 | 512 | — | — | <32 | <4 | <32 | <4 |
| K25 | N | <32 | — | <32 | — | <32 | — | <32 | — | <32 | — |
| Rho-F4M7E12 | I | <32 | — | 2048 | — | 128 | — | <32 | — | <32 | — |
| K46 | N | <32 | — | <32 | — | <32 | — | <32 | — | <32 | — |
| FM1-M37E14 | I | <32 | — | 2048 | — | 1024 | — | <32 | — | <32 | — |
| K31 | N | <32 | — | <32 | — | — | — | <32 | <4 | <32 | — |
| Oti-M179E33 | I | 128 | 128 | 128 | 128 | — | — | 2048 | 512 | 32 | <4 |
| K30 | N | <32 | — | <32 | — | <32 | — | <32 | — | <32 | — |
| Oti-M179E33 | I | 128 | — | 128 | — | <32 | — | 2048 | — | <32 | — |
| K37 | N | <32 | — | — | — | — | — | <32 | — | <32 | <4 |
| Swine 1976-M52 | I | <32 | <4 | — | 4 | — | — | <32 | <4 | 512 | 1280 |
| K38 | N | <32 | — | 32 | — | 32 | — | <32 | — | <32 | — |
| Swine 1976-M52 | I | <32 | — | 64 | — | 32 | — | <32 | — | 256 | — |

F = Ferret. M = Mouse. E = Chick embryo.
Numbers after letters correspond to number of passages in animals.
N = Normal serum. I = Immune serum.
All titers are reciprocals of one.
— = Not done. HI = Hemagglutination-inhibition titer. MN = Neutralization titer in mice.

In this respect the response of the children appeared to be clearly more specific.

A further comparison of the response of the children and the adults to the A-prime infection is listed in Table III as the per cent showing incremental change in titer. This demonstrated again greater specificity on the part of the children with a rather striking parallel in the pattern of response to the Rhodes and Oti strains. The results in the adult group were less pronounced but followed the same pattern. As previously noted vaccination tends to mask the effect of A-prime infection on the antibody responses to the PR8 strain.

It appears that the Oti strain, when tested against human sera from individuals who had experienced A-prime infection, showed antibody reactions which strongly suggest that it is an A-prime strain.

*Results of Studies Using Specific Animal Antisera.* The results in the two illness groups indicated the desirability of further investigation of the immunological characteristics of the Oti strain. Table IV shows the relation of cross-inhibition tests using specific ferret antisera against Type A, A-prime and swine strains. It is readily apparent that the Oti strain is more closely related to the A-prime and A strains than to the Swine 1976 strain. Furthermore, neutralization tests in mice paralleled the agglutination-inhibition tests. Animals inoculated with the Oti strain developed high antibody titers against the Oti strain and slightly less antibody against the Type A or A-prime strains while exhibiting no antibody against the swine strain. Animals inoculated with the A and A-prime strains had no antibody against either the Oti or the swine strain 1976. These results differed from the findings in man where experience with an A-prime infection caused rises in anti-Oti antibody. Using the more specific animal sera less evidence of cross inhibition between the Oti and other strains was obtained than between the A-prime strains of Rhodes and FM1. The Oti strain did not crossreact with the antisera to the PR8, Type A, strain. It should be pointed out, however, that anti-Oti serum gave as much inhibition of the Rhodes strain as anti-Rhodes serum did with the FM1 strain. Further, there is considerable variation in the amount of cross reaction obtainable with strains of the A-prime group. In any event, it appears that this strain behaves like a type A strain and certainly does not have the characteristics of the swine strains described by Shope.[10] These data, while not decisive, taken together with the results obtained with human sera seem to warrant the inclusion of the Oti strain with the A-prime groups of 1947.

*Discussion.* The history of the Oti strain seems sufficiently accurate to accept the fact that it was originally isolated from infected swine in Korea where swine influenza is recognized largely as a low grade endemic disease and differs from the sharp epidemics described in the United States. The data presented in this paper call attention to the definite antigenic resemblance of this swine strain, isolated in 1939, to strains such as the A-prime group first prominently encountered in human disease in 1947. It may be that some of the peculiarities of that group of strains are related to their earlier adaptation in the animal host. If subsequent studies should increase the possibility that these infections of man arose from a swine host, it would represent a reversal of the sequence of host infection from that suggested earlier, by Koen[9] and Shope[10] for swine influenza recognized in the United States. On the other hand, the virus may have been of human origin, fortuitously detected in swine.

*Conclusions.* 1. A strain of swine influenza virus (Oti), isolated in 1939, has been reinvestigated by serological means and found antigenically to resemble some members of the human A-prime influenza virus group.

2. Significant increases in the levels of antibody against this swine strain of influenza virus were detected in the convalescent blood specimens of children who had had experience during the A-prime influenza epidemic of early 1947. High titers of antibody were also found against the current A-prime strains.

3. Adults involved in the same epidemic also showed significant antibody increases against the same swine strain and the current A-prime and Type A strains.

4. Results of cross hemagglutination inhibition tests with specific ferret anti-sera supported the observations made from the serological tests with human sera.

5. The relationship of this swine strain to members of the A-prime group has been discussed.

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[9] Dorset, M., McBryde, C. N., and Niles, W. B., *J. Am. Vet. Assn.*, 1922-23, **62**, 162.

[10] Shope, R. E., *Harvey Lectures*, 1935-36, **31**, 183.

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## 17140. The Hyperglycemic Action of Some Analogs of Epinephrine

EVAN W. MCCHESNEY, JOHN P. MCAULIFF, AND HAROLD BLUMBERG.*

*From the Sterling-Winthrop Research Institute, Rensselaer, N. Y.*

The preparation in the chemical laboratories of this Institute of a series of compounds to be studied for bronchodilator action has made available to us a group of epinephrine analogs, all of which have this action to a desirable degree. They also lack some of the undesirable side effects of epinephrine, notably the abrupt pressor action.[1-3] The marked hyperglycemic action of epinephrine would also constitute an undesirable side effect, particularly if it were to be used for the treatment of bronchial asthma in diabetics. It was therefore of interest to determine whether these compounds have appreciable hyperglycemic actions, and the results of the study are included in this report. The recent resolution of arterenol (dl-norepinephrine) by Tullar[4] has provided the opportunity to study the pharmacology[5] and the hyperglycemic effects of the two optical isomers of this compound also. 1-Arterenol is of particular interest because of its possible identity with Sympathin E.[6-8] The compounds studied and their structural formulae are given below:

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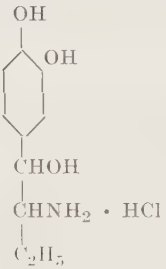
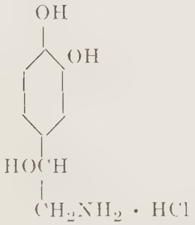
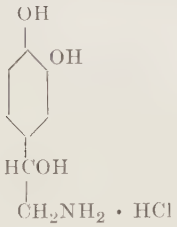
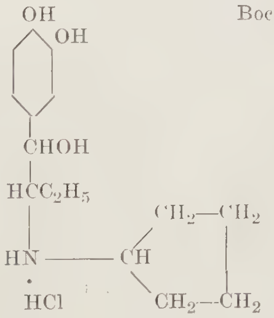
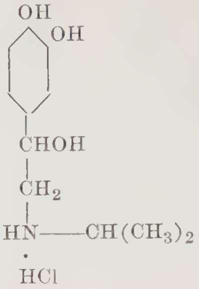
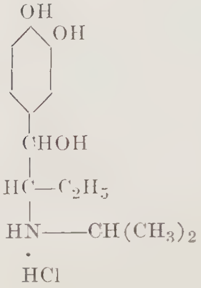
* Present address: Endo Products Inc., Richmond Hill, N. Y.

| Compound No.* | Chemical or trade name | Structural formula | Reference to method of preparation |
|---|---|---|---|
| Win 162 | Butanefrine† hydrochloride (dl-ethylnorepinephrine hydrochloride) |  | Suter and Ruddy[9] |
| Win 273-6 | l-Arterenol hydrochloride |  | Tullar[4] |
| Win 273-5 | d-Arterenol hydrochloride |  | Tullar[4] |
| Win 515 | dl-1-(3,4-dihydroxyphenyl)-2-cyclopentylaminobutanol hydrochloride |  | Bockmühl *et al.*[10] |

| | | | |
|---|---|---|---|
| Win 516-2 | Isuprel† hydrochloride [dl-1-(3,4-dihydroxyphenyl)-2-isopropylamino-ethanol-hydrochloride] |  | Scheuing and Thomä[11] |
| Win 3046 | dl-1-(3,4-dihydroxyphenyl)-2-isopropylamino-1-butanol hydrochloride |  | Bockmühl *et al.*[10] |

* Laboratory system of classification.

† Registered trade-marked names of Winthrop-Stearns Inc.

*Experimental methods.* The compounds to be tested were injected subcutaneously into healthy adult rabbits (wt. 1.5-4 kg), following an 18 hr fast. They were administered in a volume of 1 cc of 0.9% NaCl/kg except in cases where solubility limitations required a larger volume. Control animals received the saline injection only. Blood samples were taken from the ear vein and were analyzed for glucose by the Folin-Malmros method,[12] or by a slight modification† of the Schales and Schales method.[13] Samples were usually taken at 1, 2, 3, and 5 hours after the injection. Analyses were also made before the injection, and any animal which gave a fasting blood sugar level of 130 mg% or more was rejected from that test. The results are presented in Table I. For purposes of simplification of the Table, only the peak rise of blood sugar, the extreme range of the rises in the several animals, and the approximate time of

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[1] Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M., *J. Pharm. and Exp. Therap.*, 1948, **92**, 369.

[2] Tainter, M. L., Cameron, W. M., Whitsell, L. J., and Hartman, M. M., *ibid.*, 1944, **81**, 269.

[3] Lands, A. M., Siegmund, O., and Ananenko, E., *Fed. Proc.*, 1949, **8**, 312.

[4] Tullar, B. F., *J.A.C.S.*, 1948, **70**, 2067.

[5] Ludueña, F. P., Ananenko, E., Siegmund, O., and Miller, L. C., *J. Pharm. and Exp. Therap.*, 1949, **95**, 155.

[6] Gaddum, J. H., and Goodwin, L. G., *J. Physiol.*, 1947, **105**, 357.

[7] West, G. B., *Pharm. J.*, 1947, **158**, 6.

[8] von Euler, U. S., *Science*, 1948, **107**, 422.

[9] Suter, C. M., and Ruddy, A. W., *J. A. C. S.*, 1944, **66**, 747.

[10] Bockmühl, M., Ehrhart, G., and Stein, L., U. S. Patent No. 2,083,001.

[11] Scheuing, G., and Thomä, O., U. S. Patent No. 2,308,232.

[12] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115; Horvath, S. M., and Knehr, C. A., *ibid.*, 1941, **140**, 869.

† The authors state simply that a Folin-Wu filtrate is to be used. The filtrate was therefore prepared as follows: to 0.2 cc blood add 3.8 cc water followed by 3 cc of 0.67% sodium tungstate and 3 cc of 0.045 N. sulfuric acid. Centrifuge, and take 5 cc for analysis. If the blood sugar is found to exceed 300 mg %, the analysis is repeated on 2 cc of filtrate.

[13] Schales, O., and Schales, S. S., *Arch. Biochem.*, 1945, **8**, 285.

TABLE I.
Hyperglycemic Action of Some Epinephrine Analogs in the Rabbit.

| Compound name or No. | Dose,* mg/kg | No. of rabbits | Rise in blood sugar, mg% Avg | Range | Time of peak rise, hr after inj. |
|---|---|---|---|---|---|
| l-epinephrine | 0.15 | 3 | 106 | 18-157 | 3 |
| '' | 0.3 | 3 | 207 | 200-212 | 3 |
| '' | 0.5 | 3 | 495 | 462-552 | 2,4§ |
| Win 162 | 1 | 3 | 11 | 7-14 | 2† |
| '' | 2 | 3 | 20 | 14-31 | 2 |
| '' | 5 | 3 | 50 | 41-65 | 2 |
| '' | 10 | 6 | 76 | 56-96 | 2 |
| l-arterenol | 0.25 | 2 | 40 | 23-57 | 1 |
| '' | 0.5 | 4 | 35 | 18-44 | 2§ |
| '' | 1 | 3 | 96 | 53-149 | 3 |
| '' | 2 | 6 | 176 | 52-230 | 2 |
| d-arterenol | 2 | 4 | 17 | 5-31 | 2 |
| '' | 10 | 8 | 47 | 0-86 | 2-3‡ |
| Win 515 | 10 | 4 | 61 | 43-106 | 1-2‡ |
| '' | 20 | 6 | 83 | 25-145 | 1-3‡ |
| Isuprel | 10 | 10 | 18 | 0-37 | 1-4‡§ |
| '' | 25 | 6 | 28 | 0-45 | 1§ |
| Win 3046 | 5 | 5 | 52 | 20-85 | 1 |
| '' | 10 | 5 | 84 | 40-140 | 1-2‡ |
| '' | 20 | 6 | 71 | 36-98 | 1 |
| Controls | 0 | 8 | 2 | —2-10 | — |

* All doses were calculated in terms of the free base.

† After it was established preliminarily that the peak response was at 2 hours after injection the subsequent observations were made only at that time.

‡ In these cases about as many animals showed peak responses at one time as another.

§ No observation was made at 3 hours in these cases.

this average peak are recorded in the Table.

*Conclusions.* Of the several compounds tested, only l-arterenol has a hyperglycemic action of the same order as that of l-epinephrine. Its activity can be placed at about ⅛ of that of epinephrine, while the d-isomer has about 1/20 of the activity of the l-isomer. Sahyun[14] found that dl-arterenol, 1 mg/kg, produced an average rise in blood sugar of 78 mg% 3 hrs after subcutaneous injection in 7 rabbits. This is considerably more activity than would be calculated from our observations on the separate isomers. However, if one of his rabbits (No. 8, Table II) is eliminated from consideration, the average rise becomes only 61 mg%, and the discrepancy is much less. Of the other compounds studied, Isuprel is clearly the least active. The approximate hyperglycemic activity of the whole series of compounds, taking l-epinephrine as 100, is as follows (all figures given have been calculated in terms of the free base): l-arterenol, 12; Win 3046, 1.7; Butanefrine, 1.6; Win 515, 0.7; d-arterenol, 0.6; and Isuprel, 0.12.

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[14] Sahyun, M., *Arch. Intern. Pharm.*, 1933, **45**, 285.

# 17141. Further Investigations on *Donovania granulomatis.*

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It has been suggested elsewhere[1,2] that *Donovania granulomatis,* the etiological agent of granuloma inguinale,[3] may be a member of the tribe *Eschericheae* and be related to the genus *Klebsiella.* In this connection it was of interest to examine the carbohydrate fermentation reactions of *D. granulomatis.* Members of the *Klebsiella* group are known to vary widely in their capacity to ferment sugars, the *K. pneumoniae* group being the most active and *K. rhinoscleromatis* the least.

For the present investigation both solid and fluid media were used. These have been described in detail elsewhere.[4] In brief, the fluid medium was a beef heart infusion broth without the usual added glucose but with the various sugars listed in Table I added to a final concentration of 1% and with Andrade's indicator. The solid medium was a Levinthal beef heart agar again without the usual added glucose but with the same sugars to a final concentration of 1% and with Andrade's indicator. The results are shown in Table I.

All fermentation tests were held for 4 weeks. It will be noted that in broth only the 3 monosaccharides, glucose, fructose and galactose were fermented, while on solid medium there was also a delayed fermentation of maltose. In no case was any gas produced. In general *D. granulomatis* would seem to be even less active than *K. rhinoscleromatis* but from the point of view of these biochemical reactions could fit into the pattern of the *Klebsiellae.*

TABLE I.
Fermentation Reactions of *D. granulomatis.*

| | Broth | Agar |
|---|---|---|
| Glucose | + (5)* | + (5) |
| Fructose | + (6) | + (8) |
| Galactose | + (6) | + (5) |
| Maltose | 0 | + (9) |
| Sucrose | 0 | 0 |
| Lactose | 0 | 0 |
| Mannitol | 0 | 0 |
| Inositol | 0 | 0 |

* Indicates that signs of acid formation were first noted on the 5th day.

Further studies have been carried out on the sensitivity of *D. granulomatis* to the different streptomycins. The *in vitro* sensitivity has proved to be particularly high, over 3 times as high as the next most sensitive organism. In Table II the minimum inhibiting concentrations of the pure streptomycins are shown and compared to the next most sensitive organism encountered—the BCG strain of *M. tuberculosis* var. bovis—and to *K. pneumoniae,* an organism of greater than average sensitivity.

*Summary. D. granulomatis* shows delayed fermentation of a few carbohydrates, chiefly monosaccharides, without gas formation. In this respect it resembles *K. rhinoscleromatis.* Its sensitivity is high against all the pure streptomycins and cases of granuloma inguinale should react as favorably to treatment with dihydrostreptomycin as to treatment with streptomycin itself.

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TABLE II.
*In vitro* Sensitivity Against Pure Streptomycins.

| Organism | Streptomycin | Dihydrostreptomycin | Mannosidostreptomycin | Dihydromannosidostreptomycin |
|---|---|---|---|---|
| *D. granulomatis* | 0.167 | 0.151 | 0.595 | 0.446 |
| *K. pneumoniae* | 1.76 | 1.76 | 6.39 | 6.59 |
| *M. tuberculosis* (strain BCG) | 0.52 | 0.55 | 1.9 | 1.7 |

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[1] Rake, G., *Am. J. Syph., Gon. and Ven. Dis.,* 1948, **32**, 150.

[2] Rake, G., *J. Bact.,* 1948, **55**, 865.

[3] Anderson, K., *Science,* 1943, **97**, 560.

[4] Rake, G., and Oskay, J. J., *J. Bact.,* 1948, **55**, 667.

# 17142. Effect of Cortical Extract and Other Agents on Eosinophilia of Mice Infected with *Trichina spiralis*.*

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An absolute reduction in the number of circulating eosinophiles occurs within a few hours following the stimulation of the human adrenal cortex with adrenotrophic hormone.[1] The same effect can be induced by the administration of cortical hormone, or epinephrine[2] which presumably stimulates anterior pituitary secretion of adrenotrophic hormone. Lymphopenia accompanies the drop in eosinophiles.[3]

A test animal in which eosinopenia can be readily determined for bioassay of adrenotrophic activity is desirable. In the mouse the normal level of blood eosinophiles is so low that normal animals cannot be utilized to good advantage. However, within 3-4 weeks after forcible ingestion of trichina-infected meat the mouse exhibits a decided eosinophilia which persists at a rather constant level for a matter of weeks. It was the purpose of this investigation to determine whether such infected animals might serve as a test object for hematologic assay of adrenocortical activity and/or function. The procedure was the following:

1. Young adult male mice were force-fed pectoral muscle from trichinous hosts (infected rats);‡ eosinophilia of 4-20% was present within 4 weeks and persisted for at least 5 weeks.

2. Total and differential counts, the latter on at least 100 cells, stained with Giemsa or Wright's stain, were made on the tail blood of unanesthetized animals.

3. The test material was then administered and counts were made after 6 and 24 or 48 hours.

The materials tested for their effect on the trichina-induced eosinophilia were whole adrenal cortex in oil, testosterone propionate, estradiol dipropionate, epinephrine, urethane, and peanut oil.§ Crystalline hormones were dissolved in peanut oil, urethane in water.

Within 6 hours after the subcutaneous administration of cortical extract (0.1 cc lipo-adrenal cortex—Upjohn) the percentage of circulating eosinophiles had dropped from 12.7 to 1.6 (average of 7 animals). In this series of animals the decline in total white blood cell count averaged 5700 cells/mm$^3$, in eosinophiles 1800, and in lymphocytes 2900. The initial counts averaged 16,400. Preliminary findings on 4 intact and 2 adrenalectomized trichinous mice injected subcutaneously with sex steroids (70 $\mu$g testosterone propionate and 25 $\mu$g estradiol dipropionate) revealed that within 6 hours following injection, except in one animal, the percentages of the white blood cells were not significantly affected. This was true also for the one adrenalectomized and 4 intact trichinous mice which received injections of peanut oil.

The absolute number and the percentage of circulating eosinophiles were reduced in trichinous mice by the subcutaneous administration of epinephrine (0.02 mg/100 g hourly for 4 hours). The drop in eosinophiles (at

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* This investigation has been aided by grants from the Donner Foundation and the Graduate School of the University of Minnesota.

† Present address: Department of Zoology, Mount Holyoke College, South Hadley, Mass.

[1] Thorn, G. W., Forsham, P. H., Recant, L., and Hills, A. G., *Program Assn. Study Internal Secretions*, 1948, 19.

[2] Recant, L., Forsham, P. H., and Thorn, G. W., *Program Assn. Study Internal Secretions*, 1948, 19.

[3] Hills, A. G., Forsham, P. H., Finch, C. A., *Blood*, 1948, **3**, 755.

‡ The author is indebted to Dr. Franklin Wallace of the Department of Zoology, University of Minnesota, for supplying rats infected with trichina.

§ The author is indebted to Dr. F. E. Houghton of Ciba Pharmaceutical Products, through whose courtesy the sex hormones were made available, and to Dr. L. E. Josselyn of Abbott Laboratories for supplying urethane.

6 hours) paralleled that produced by cortical hormone. The percentage of circulating eosinophiles dropped from 14.3 to 2.4, the per cent of lymphocytes remaining unaltered.

The absolute number of eosinophiles dropped from 2700 to 400 cell/mm$^3$. In 2 adrenalectomized trichinous mice which received epinephrine, the total count had doubled within 6 hours. The percentages of eosinophiles and the other types of cells remained the same.

Urethane in aqueous solution (1 mg or ½ mg per g body weight) was injected into 6 intact trichinous mice. After 6 hours the absolute number of lymphocytes had dropped to approximately half, the eosinophiles to less than half the initial levels. Much greater decreases were obtained in 6 mice which survived 5 or 6 daily anesthetic doses of urethane. The percentage of eosinophiles dropped from 9.3 to 2.3, the absolute number from 1800 to 100. The absolute number of lymphocytes dropped from 12600 to 2200.

Urethane-induced lymphopoenia has been observed in adrenalectomized as well as intact rats and mice.[4,5] Urethane depressed the white blood cell count in mouse myeloid leukemia, but adrenal cortical extract was ineffective.[6] These observations indicate that the effect of urethane on normal blood lymphocytes and leukemic myeloid cells is not mediated by the adrenal cortex. In the present experiments eosinopoenia paralleled lymphopoenia following the injection of urethane. Adrenalectomized trichinous mice did not tolerate anesthetic doses of urethane, and in these experiments it could not be determined whether depression of blood eosinophiles occurs as an effect of urethane in the absence of the adrenal cortex. Spink[7] demonstrated that reduction in the eosinophile count of trichinous guinea pigs occurred with a superimposed bacterial infection. The eosinophile count was also suppressed in a patient developing bacterial infection during the course of trichinosis.

*Summary.* The number of circulating eosinophiles of trichinous mice was decidedly reduced by the administration of cortical hormone. The same effect was induced by epinephrine in intact, but not in adrenalectomized, trichinous mice. Sex steroids in the doses used did not significantly reduce the percentage of circulating eosinophiles in 4 intact and 2 adrenalectomized trichinous animals. Eosinopoenia was induced by urethane in trichinous mice. Whether or not urethane-induced eosinopoenia is mediated by the adrenal cortex has not been determined.

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[4] Dury, A., and Robin, E. D., *Endocrinology*, 1948, **42**, 320.

[5] Lu, C. S., Thesis, University of Minnesota, 1947.

[6] Lu, C. S., Kirschbaum, A., *Proc. Internat. Cancer Res. Congress*, 1947, 105.

[7] Spink, W. W., *Arch. Int. Med.*, 1943, **54**, 805.

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## 17143. Effect of Pteroylglutamic Acid and Vitamin $B_{12}$ on Growth of Rous Tumor Implants.

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Earlier work from this laboratory showed that pteroylglutamic acid was essential for the growth of implants of Rous sarcoma in young chicks.[1] The effect of other dietary factors was much less pronounced.[2] A search was then made for additional dietary factors which might affect the growth of this sarcoma in chicks.

Day-old chicks in groups of 10 were fed

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[1] Little, P. A., Sampath, A., Paganelli, V., Locke, E., and SubbaRow, Y., *Tr. N. Y. Acad. Sci.*, Series II, 1948, **10**, 91.

[2] Little, P. A., Oleson, J. J., and SubbaRow, Y., *J. Lab. and Clin. Med.*, 1948, **33**, 1139.

TABLE I.
Comparative Effects of Pteroylglutamic Acid, Liver Extract and Vitamin $B_{12}$ on the Growth of Rous Sarcoma.

| Supplements | Amt inj. daily | No. of tests | Total No. of chicks | % chicks w. tumors | Avg tumor size |
|---|---|---|---|---|---|
| None | — | 6 | 60 | 3 | + |
| Pteroylglutamic Acid | 5 $\gamma$ | 3 | 23 | 61 | + |
| ,, ,, | 10 $\gamma$ | 5 | 50 | 78 | ++ |
| ,, ,, | 50 $\gamma$ | 6 | 57 | 88 | +++ |
| 15 Unit Liver | .01 cc | 1 | 10 | 70 | + |
| 15 ,, ,, | .025 cc | 2 | 20 | 60 | + |
| 15 ,, ,, | .05 cc | 2 | 20 | 60 | ++ |
| 15 ,, ,, | .10 cc | 2 | 20 | 85 | +++ |
| Vit. $B_{12}$ concentrate | $\cong$ .05 $\gamma$ | 2 | 20 | 55 | + |
| ,, ,, ,, | $\cong$ 0.1 $\gamma$ | 1 | 10 | 60 | ++ |
| ,, ,, ,, | $\cong$ 0.5 $\gamma$ | 2 | 20 | 75 | +++ |
| ,, ,, ,, | $\cong$ 1.0 $\gamma$ | 2 | 19 | 68 | +++ |
| ,, ,, ,, | $\cong$ 2.0 $\gamma$ | 2 | 20 | 85 | +++ |
| Vit. $B_{12}$<br>Pteroylglutamic Acid | $\cong$ 0.5 $\gamma$<br>10 $\gamma$ | 1 | 10 | 90 | ++++ |
| Vit. $B_{12}$<br>Pteroylglutamic Acid | $\cong$ 2.0 $\gamma$<br>10 $\gamma$ | 1 | 10 | 100 | ++++ |

the synthetic pteroylglutamic acid deficient diet and water *ad libitum* and were injected with tumor suspension as previously described.[2] Supplements of pteroylglutamic acid and the preparations reported here were injected intramuscularly daily at the levels indicated in the table.

It was found that 15 unit liver extract* would apparently replace pteroylglutamic acid at levels as low as 0.01 ml per day in stimulating the growth of Rous sarcoma in chicks fed this synthetic diet. It is known that parenteral liver extracts contain very little pteroylglutamic acid[3] and therefore cannot substitute for this factor in the nutrition of chicks. A preliminary study was then made of the properties of the factor in liver which was responsible for tumor stimulation.

Since 15 unit liver extract (beef) is a rich source of vitamin $B_{12}$ and the properties of the liver factor were found to be similar to this vitamin, highly purified concentrates of vitamin $B_{12}$ were also tested. These were found to give the same results as the liver extract itself. The preparations were previously assayed microbiologically† and by a chick growth method[4] for their vitamin $B_{12}$ content.

Some typical results are presented in Table I. Pteroylglutamic acid alone gives a high incidence of growing tumors of good size. The liver extract alone gives quite similar results. The effect of the liver is duplicated by dosages of vitamin $B_{12}$ as low as 0.05 $\gamma$ per day. The higher levels of this factor give larger sized tumors than the lowest levels in the 14-day test period. When pteroylglutamic acid and vitamin $B_{12}$ are given together, the tumors come up earlier and attain greater size than with corresponding levels of either factor alone.

Thus it appears that vitamin $B_{12}$ does not entirely replace pteroylglutamic acid in its growth promoting effect on the Rous tumor but is required in addition to give maximum tumor growth under these particular dietary conditions.

*Summary.* Vitamin $B_{12}$ has been demonstrated to have a role in the growth of Rous sarcoma in chickens comparable to that previously shown for pteroylglutamic acid.

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* 15 Unit liver-Lederle (prepared from beef liver).

[3] Clark, G. W., *Am. J. Med. Sci.*, 1945, **209**, 520.

† We thank Mr. A. C. Dornbush for these assays.

[4] Whitehill, A. R., Wieland, O., and Oleson, J. J., unpublished data.

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## 17144. Adsorption of Urinary Gonadotrophins on Kaolin.

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Zondek[1] described a method of obtaining gonadotrophic hormones from urine by precipitation with 4 volumes of alcohol. With minor modifications, such as the substitution of acetone for alcohol, this has remained the basic method of preparing urinary gonadotrophic hormones for assay.[2-5] Other procedures have been less commonly employed. Katzman and Doisy[6] recovered the hormone from urine by precipitation with acetone saturated with benzoic acid. Since the hormone is a protein, precipitating agents such as tannic acid[7] and ammonium sulphate[8] have been used, and ultrafiltration[9,10] has also proved successful. Katzman, *et al.*,[11] demonstrated that chorionic hormone could be adsorbed from urine by passing it through a column of permutit. Scott[12] and later Landgrebe and Samson[13] used kaolin as the adsorbing agent for chorionic hormone.

Since chorionic hormone could be adsorbed on permutit or kaolin it seemed probable that the pituitary gonadotrophin could also be recovered from urine by an adsorption technic. This report describes a procedure by which either chorionic or pituitary gonadotrophins can be recovered from urine. The main advantages are, one, a great saving in alcohol over that which has been used previously and, two, a shorter time for preparation since dialysis is not necessary.

*Materials and method.* Kaolin (N.F. powder), that has been washed with normal hydrochloric acid and then with water until neutral, is made up as a 20% suspension in water. This is a stable stock preparation that should be shaken thoroughly before using.

1. Use a fresh 12 or 24 hour specimen of urine. Acidify to pH 4.5 with acetic (or hydrochloric) acid and then run through a coarse filter to remove mucus or particulate matter.

2. Add 5 volumes percent of the kaolin suspension to the acidified urine and stir thoroughly. Allow the kaolin to settle out. (One hour is usually sufficient but the mixture may be set in the refrigerator overnight).

3. Decant the supernatant and collect the sediment in a large centrifuge flask. Centrifuge, and discard the supernatant.

4. Add water and resuspend the kaolin by vigorous stirring or shaking. Centrifuge or allow to settle, decant and wash again with water until the kaolin is free of urine. This washing replaces the dialysis of the alcohol method.

5. Elute the hormone from kaolin by adding a volume of normal ammonium hydroxide equal to that of the kaolin suspension originally added to the urine. Stir thoroughly and allow to stand 20 to 30 minutes. Centrifuge and save the supernatant. The kaolin may be washed again, the second ammonia eluate added to the first and the kaolin discarded.

6. Add glacial acetic acid to the combined eluates until pH 8.5 is reached. If a flocculent precipitate forms, centrifuge and discard.

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[1] Zondek, B., *Zentralbl. f. Gynak.*, 1929, **53**, 834.

[2] Bellerby, C. W., *Nature*, London, 1934, **133**, 494.

[3] Crew, F. A. E., *Brit. Med. J.*, 1939, 766.

[4] McCullagh, D. R., and Bowman, W. E., *Endocrinology*, 1940, **27**, 525.

[5] Heller, C. G., and Chandler, R. E., *J. Clin. Endocrinol.*, 1942, **2**, 252.

[6] Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, 1932, **98**, 745.

[7] Levin, L., and Tyndale, H. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 516.

[8] Jones, G. E. S., and Bucher, N. L. R., *Endocrinology*, 1943, **32**, 46.

[9] Gorbman, A., *Endocrinology*, 1945, **37**, 177.

[10] Jungck, E. C., Maddock, W. O., and Heller, C. G., *J. Clin. Endocrinol.*, 1947, **7**, 1.

[11] Katzman, P. A., Godfrid, M., Cain, C. K., and Doisy, E. A., *J. Biol. Chem.*, 1943, **148**, 501.

[12] Scott, L. D., *Brit. J. Exp. Path.*, 1940, **21**, 320.

[13] Landgrebe, F. W., and Samson, L., *J. Obst. and Gyn. Brit. Emp.*, 1944, **51**, 133.

TABLE I.
Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. Aliquots of Pooled Specimens Were Adjusted to Different Degrees of Acidity Before Adsorption on Kaolin.

| Exp. No. | Control Alcohol ppt. | Adsorbed on Kaolin at pH 7.0 | 6.0 | 5.5 | 5.0 | 4.5 | 4.0 | 3.5 | 3.0 | Eluted with |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 69 | | | | 59 | | 19 | | 46 | 3N $NH_4OH$ |
| | 47 | | | | 58 | | 15 | | 35 | |
| 2 | — | | | | 99 | | 57 | | 17 | ” |
| | — | | | | 76 | | 73 | | 9 | |
| 3 | 136 | | | | 112 | | 101 | | 81 | 2N $NH_4OH$ |
| | 118 | | | | 82 | | 145 | | 107 | |
| 4 | — | | | 16 | 87 | 72 | 58 | 65 | | ” |
| | — | | | 19 | 62 | 87 | 66 | 71 | | |
| 5 | — | 18 | 18 | | 32 | | 39 | | 20 | ” |
| | — | 15 | 17 | | 46 | | 34 | | 18 | |

Exp. 1, 2, and 3, urine acidified with hydrochloric acid.
Exp. 4 and 5, urine acidified with acetic acid.

Bring the clarified supernatant to pH 5.5 with glacial acetic acid.

7. Add 4 volumes of 95% ethanol, slowly and with constant stirring to precipitate the hormone. Set in refrigerator until thoroughly chilled, centrifuge and dry the precipitate in a gentle stream of air.

8. Dissolve the precipitate in a volume of water or phosphate buffer (pH 8.0) suitable for assay.

In our experience, urines from postmenopausal women or from patients with other hypergonadotrophic conditions give suitable assay dose ranges in infantile female rats if the final extract is made up so that 1 cc is equivalent to one hour's output of urine. Six injections of 0.5 cc each make the total dose equal to a 3 hour urine volume. The examples given below were made up in this way and show the ovarian weights at autopsy 72 hours after the first injection. If mice are used for assay the total dose can be reduced to the equivalent of about one hour's urine output. Assays to determine the quantities of hormone present in the urine of normal men or women require the use of larger quantities per test mouse or rat so the total dose should be equivalent to a 6 or 12 hour volume.

*Proof of method.* In the course of developing and testing the above method of adsorbing urinary gonadotrophins on kaolin, the optimal conditions and quantities to be used in each step were determined. Usually the yield of hormone was compared to that obtained from an aliquot precipitated with alcohol as a standard of reference.

It has been customary to use fresh urine specimens to avoid partial or total destruction of the hormone through bacterial or chemical degradation. In three experiments the urine was allowed to stand several days and become very alkaline through the breakdown of urea to ammonia. When precipitated directly with alcohol these specimens yielded such highly toxic extracts that assays were not possible. After acidification and adsorption on kaolin, washing with water definitely reduced the toxicity of the extracts and hormone was recovered. In order to learn whether urease would affect the yield of hormone, that enzyme was added to urine and allowed to react for 2 hours with formation of ammonia. Subsequent acidification and adsorption on kaolin resulted in quantitative recovery of the hormone. The alkaline change due to urease activity is apparently not immediately destructive of the hormone. Collection of urine under toluene tends to preserve the natural acidity and the toluene is easily separated before kaolin is added.

The optimal pH for adsorption on kaolin was determined in several trials in which ali-

TABLE II.

Ovarian Weights in Immature Rats Injected with Extracts of Urine from Postmenopausal Women. The hormone was adsorbed on kaolin and then the aliquots of the kaolin were eluted with different concentrations of ammonium hydroxide as noted.

| | | Eluted from kaolin with $NH_4OH$ Normality | | | | | |
|---|---|---|---|---|---|---|---|
| Exp. No. | Control Alcohol ppt. | 3.0 | 2.5 | 2.0 | 1.5 | 1.0 | No. of elutions |
| 6 | 139 | 68 | | 117 | | 45 | 1 |
| | 128 | 41 | | 113 | | 15 | |
| | 148 | 106 | | 92 | | 16 | |
| 7 | 47 | 66 | | 101 | 83 | 24 | 2 |
| | 43 | 82 | | 71 | 90 | 19 | |
| 8 | | 147 | 169 | 145 | 177 | 139 | 2 |
| | | 151 | 183 | 146 | 140 | 165 | (After water wash) |
| | | 121 | 151 | 119 | 128 | 115 | |

quots of urine were adjusted to different degrees of acidity. In 3 experiments the pH was adjusted with hydrochloric acid (Table I) and recovery of the hormone was uniformly good at pH 5, but was poor in one instance at pH 4 and once at pH 3. In 2 other experiments using acetic acid the results were satisfactory from pH 3.5 to 5 but since the hormone was not adsorbed above or below this range, pH 4.5 has been selected as an optimal acidity for adsorption. Sulfuric acid was used in 2 experiments without success.

Having adsorbed the hormone onto kaolin, the next problem was to determine the optimal conditions for elution and recovery. Ammonium hydroxide was used in the elution process in concentrations up to 3 N without appreciable destruction or loss of hormone, (Table II). In Experiments 6 and 7 the recovery with 1 N $NH_4OH$ was incomplete as compared to higher concentrations of ammonia but in Experiment 8 it was satisfactory. Experiments 6 and 7 were done before it was found that the kaolin can be washed with water and that the residual acid in the kaolin probably partially neutralizes the ammonia and reduces its efficiency. We have failed repeatedly to recover the hormone from kaolin with NaOH in concentrations from 0.1 N up to 3 N. Since elution with ammonia after using NaOH has also failed to recover the hormone, it appears that the pituitary gonadotrophin is easily and rapidly destroyed by sodium hydroxide. By contrast, chorionic gonadotrophin is apparently eluted quantitatively from kaolin by either 0.1 N NaOH or N $NH_4OH$.

Katzman, *et al.*, used 10% ammonium acetate in 40% ethanol to elute the chorionic hormone from permutit. This mixture is also satisfactory for eluting gonadotrophin from kaolin and is superior to ammonium hydroxide in alcohol, but, in our experience is not superior to aqueous ammonia. There are no experiments in which the dry weights of the final precipitate can be compared to give an indication of which eluting mixture may give the higher potency per mg of precipitate. The relative effectiveness of ammonium hydroxide in water and ammonium acetate in alcohol suggested that ethanolamine might be an effective elutant; this was proved in two experiments but seemed to offer no advantage over either of the others.

Other adsorbents were tried only casually. In three experiments the hormone was adsorbed on Filtercel and then recovered with ammonia. The coarser particles of permutit adsorbed the hormone more effectively when packed in a column and the urine allowed to percolate rather slowly. Charcoal (Norit) adsorbed the hormone but no eluting agent was found.

The quantity of kaolin necessary to provide a surface area adequate for complete adsorption and still have a minimal volume for elution procedures was determined in several experiments (Table III). In experiment 9, 1 volume percent (15 cc in 1500 cc of urine) of kaolin suspension gave as complete recovery of hormone as 10 volumes percent. However, in experiment 10, the smaller quantities of kaolin were less effective. On

TABLE III.
Ovarian Weights of Immature Rats Injected with Extracts of Urine from Postmenopausal Patients. The amount of kaolin added to the urine was varied as indicated to determine the minimal amount of kaolin that would adsorb the hormone.

| Exp. No. | Control Alcohol ppt. | Quantity of kaolin suspension Volumes % 10 | 3 | 1 |
|---|---|---|---|---|
| 9 | — | 130 | 107 | 140 |
| | | 112 | 68 | 153 |
| 10 | 55 | 91 | 53 | 18 |
| | 64 | 63 | 49 | 18 |

the basis of these observations it seems that 5 to 10 volumes percent of kaolin suspension is best for routine use.

This method has been found equally applicable to recovery of chorionic hormone or of the pituitary hormone from the urine of postmenopausal women. In 2 experiments, these hormones were adsorbed simultaneously and the expected augmentation was demonstrated by assay of the final extract. One-half of a postmenopausal urine specimen was adsorbed on kaolin (a), while 100 cc of pregnancy urine was added to the other half before addition of the kaolin (a plus b). A second 100 cc portion of the pregnancy urine was also treated with kaolin (b). In the subsequent assays, the pituitary gonadotrophin (a) induced rat ovarian weights of 52 and 58 mg, whereas, those for the pregnancy urine extract (b) were 34 and 36 mg (with corpora lutea), and those of the combined urine eluate (a plus b) were 180 and 176 mg. This represented a definite augmentation reaction resulting from the simultaneous adsorption of the pituitary and chorionic gonadotrophins. Another example may be cited: a 19-year-old boy with infantilism associated with testicular agenesis had high urinary levels of pituitary gonadotrophins (kaolin extract of 6 hour urine volume induced ovarian weights of 52 and 58 mg). He was given chorionic gonadotrophin, and on the third day of treatment, excretion of the administered hormone together with the pituitary gonadotrophin was determined by assay. The same urinary equivalent of kaolin extract (6 hours output per test rat) produced ovarian weights of 257 and 260 mg.

This study was primarily a series of spot checks to determine the limits within which a routine procedure could be developed. Small numbers of rats were used in each test since quantitative precision was not attempted. In 12 tests the extracts of alcohol precipitates were compared with those of kaolin adsorbates. In 3 instances material obtained by the alcohol method produced significantly greater increases in ovarian weights, in 6 trials the kaolin method seemed superior, and there was no significant difference in the other three. The sum of the ovarian weights for the 26 rats receiving extracts of alcohol precipitate was 2081 mg, as compared to 2073 mg for the 26 rats receiving kaolin extracts. Thus, there is no significant difference in the recovery of hormone by the two methods.

The main advantage of the kaolin method is the marked saving of ethanol. For example, the hormone from 1000 cc of urine can be adsorbed upon 50 cc of kaolin suspension, then eluted in two 50 cc portions of N ammonium hydroxide, which on subsequent acidification will not exceed 120 cc in volume and from which the hormone can be precipitated with 500 cc of alcohol. The original urine specimen would have required 4000 to 5000 cc of alcohol plus an additional quantity used in the second precipitation (after dialysis). A second advantage is that washing the kaolin with water accomplishes the same detoxification as dialysis but much more rapidly and avoids the transfer of solution to, and from, the dialysing bag.

Another advantage is that the same urine specimen can be used for both gonadotrophic and steroid determinations if the supernatant urine and the water washings from the kaolin are saved. A series of tests has shown that 17 ketosteroid estimations after the use of kaolin are the same as those obtained from untreated aliquots of urine.

*Summary.* A method is described for the adsorption of gonadotrophic hormones from urine onto kaolin and their subsequent elution with ammonium hydroxide. The relatively small volume of the eluate reduces the amount of alcohol needed to precipitate the hormone from solution, the final extracts are less toxic and can be prepared in a shorter time. The hormone assays are comparable to those obtained with the usual alcohol precipitation method.

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## 17145. Streptomycin-Producing Capacity of Different Strains of *Streptomyces griseus*.*†

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Since the first announcement[1] that certain strains of *Streptomyces griseus* produce the antibiotic streptomycin, pertinent questions have been raised in regard to the ability of this organism to produce the particular antibiotic: 1. Is this a characteristic property of all strains of the species *S. griseus?* 2. How widely distributed is the capacity for production of streptomycin among actinomycetes? 3. Did the original strain of *S. griseus,* first isolated in this country in 1915 by Waksman and Curtis,[2] also possess the capacity for producing streptomycin? (The last question is of considerable theoretical and historical interest, since it has a bearing upon the identity of the streptomycin-producing strains isolated in 1943 with the original 1915 isolate, as well as upon the fact that had the subject of antibiotics been recognized at that time, it would not have taken that long to recognize the actinomycetes as highly important producers of antimicrobial agents.)

The first two questions were soon answered. It was established[3] that only certain few strains of *S. griseus* are capable of producing streptomycin, whereas most of the other strains that can easily be isolated from natural substrates are either unable to produce any antibiotics at all or may give rise to antibiotics other than streptomycin, such as grisein,[4] and that certain actinomycetes, other than *S. griseus,* also possess the capacity of forming streptomycin.[5]

In an attempt to answer the third question, the original 1915 culture, which has been kept alive by frequent transfers since its isolation in the collection of the New Jersey laboratories under the ATCC number 3326, was tested for its antibiotic-producing capacity. The same culture was deposited in 1920 in the Baarn collection in Holland; it recently was obtained from that collection and given the number 3326a. Repeated tests for their antibiotic-producing capacity revealed the fact that the culture kept in New Jersey was totally unable to produce either streptomycin or any other antibiotic substance, whereas the corresponding Baarn culture was capable of forming an antibiotic agent, which was different, however, from streptomycin.[4] This difference may have been due to the fact that in the New Jersey collection the culture was kept on synthetic media and in the Baarn collection organic media are used. The difference pointed definitely to changes in the antibiotic-producing capacity during the 30-year period that the culture has been kept in the two collections.

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Microbiology.

† Partly supported by a grant made by the Rutgers Research and Endowment Foundation.

[1] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

[2] Waksman, S. A., and Curtis, R. E., *Soil Sci.*, 1916, **1**, 99.

[3] Carvajal, F., *Mycologia*, 1946, **38**, 596; Waksman, S. A., Reilly, H. C., and Johnstone, D., *J. Bact.*, 1946, **52**, 393.

[4] Waksman, S. A., Reilly, H. C., and Harris, D. A., *J. Bact.*, 1948, **56**, 259.

[5] Johnstone, D. B., and Waksman, S. A., *J. Bact.*, 1948, **55**, 317.

Recently, Kelner[6] also examined the original *S. griseus* culture No. 3326. He confirmed the observation that the New Jersey strain is largely inactive antibiotically. When x-rayed conidia of this organism were plated out on various media and tested against bacteria, however, several active mutants were obtained, one of which apparently produced streptomycin. Comparison of the antibiotic spectrum of this mutant with that of known streptomycin-producing strains of *S. griseus* showed these spectra to be identical.

These observations appeared to be so important that an effort was made to confirm them and to demonstrate definitely that the antibiotic produced by Kelner's mutant No. 1 was actually streptomycin. This mutant, designated as 3326b, was streaked out on plates containing suitable agar media and, after 24- and 48-hour incubation periods, various test bacteria were cross-streaked toward it. An antibiotic spectrum characteristic of streptomycin-producing strains of *S. griseus* was obtained, as shown in Table I. There was only limited growth of the streptomycin-resistant strains of *E. coli*, and there was good growth of streptomycin-dependent strains. This fully confirmed the streptomycin-producing capacity of Kelner's mutant. The presence of a narrow zone of inhibition of the streptomycin-dependent culture points to the formation of another antibiotic other than streptomycin, a fact well established for the various streptomycin-producing cultures.

TABLE I.
Antibiotic Spectrum of Mutant 1 of 1915 *S. griseus* Culture (3326b), as Determined by Agar Cross-streak Method.
Zone of inhibition in millimeters.

| Test organism | Nutrient agar | Nutrient glucose agar |
|---|---|---|
| *Escherichia coli* | 24 | 19 |
| ,, ,, sr* | 3 | 7 |
| ,, ,, sd* | 6-25† | 7-22† |
| *Bacillus mycoides* | 24 | 21 |
| *Staphylococcus aureus* | 22 | 15 |
| *Bacillus subtilis* | 28 | 23 |

*sr—streptomycin-resistant; sd—streptomycin-dependent.

†First figure—inhibition zone; second figure—growth zone.

To confirm definitely the fact that the antibiotic produced by the mutant of the 1915 culture was streptomycin, it was necessary to produce this antibiotic in suitable culture media, isolate it, concentrate it and test it. This was carried out by the usual procedures developed for streptomycin.[1] The results presented in Table II prove definitely that the antibiotic produced by the mutant of the 1915 culture (3326b) is identical with that of the 1943 isolate of the streptomycin-producing culture (3463). When varying amounts of the metabolite solutions of the two cultures were added to agar media and the latter streaked with various test bacteria, similar results were obtained, except for some minor quantitative differences. After 5 days' incubation a cup assay of 111 μg of streptomycin for the 3326b culture gave 150 *E. coli* dilution units, 250 *B. mycoides* units, 150 *S. aureus* units and 1,500 *B. subtilis* units; the corresponding results for 110 cup assay units for 3463 were 150, 500, 250 and 1,000, which were very close to the foregoing if one recognizes the limitations of this method of testing. The isolated and concentrated streptomycin preparations obtained from the two cultures also gave nearly identical results.

When streptomycin-dependent strains of *E. coli* were streaked on media containing the two preparations, comparable growth was obtained from both preparations and was very similar to growth with similar amounts of pure streptomycin added to other plates. This was true of both the culture filtrates and the concentrates obtained from them. The dependent strains of *E. coli* were affected alike by both preparations, whereas the resistant strains were not affected at all by either.

Other supporting evidence bearing upon the

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[6] Kelner, A., *J. Bact.*, 1949, **57**, 73.

TABLE II.
Identity of Antibiotic Produced by Culture 3326b with That of Streptomycin Produced by Known Streptomycin-producing Culture 3463.

| Incubation, days | Streptomycin, µg/ml | Dilution units per ml | | | |
|---|---|---|---|---|---|
| | | *E. coli* | *B. mycoides* | *S. aureus* | *B. subtilis* |
| | Culture filtrate of 1915 mutant (3326b) | | | | |
| 3 | 59 | 75 | 150 | 75 | 500 |
| 4 | 108 | 100 | 250 | 250 | 750 |
| 5 | 111 | 150 | 250 | 150 | 1,500 |
| | Crude isolated preparation of 3326b | | | | |
| | 867* | 1,500 | 3,000 | 1,500 | >10,000 |
| | Culture filtrate of streptomycin-producing culture (3463) | | | | |
| 3 | 41 | 30 | 100 | 75 | 300 |
| 4 | 64 | 75 | 150 | 75 | 750 |
| 5 | 110 | 150 | 500 | 250 | 1,000 |
| | Crude isolated preparation of 3463 | | | | |
| | 770* | 1,000 | 3,000 | 500 | 10,000 |

* Culture filtrate adsorbed on Norite, dried, eluted with acid alcohol, treated with ether, to yield aqueous concentrate.

problem under consideration may be reported here. It had been suggested previously[7] that the sensitivity of streptomycin-producing strains of *S. griseus* to a specific actinophage could be used for establishing the capacity of such strains to form streptomycin. The original 1915 culture of *S. griseus* (3326) was not sensitive to this particular phage; this was taken to indicate that there was a definite correlation between phage sensitivity of *S. griseus* strains and ability to form streptomycin, a fact confirmed by a lack of sensitivity to phage by other, freshly isolated, non-streptomycin-producing strains of *S. griseus*. When Kelner's mutant was tested for sensitivity to this phage, it was found to be highly sensitive. This is illustrated in Table III. The streptomycin-producing, including the two original isolates (3463, 3464), a spore isolate of one of these (3464a), and a freshly isolated culture (3481), were all phage-sensitive as measured by the rate of cell multiplication. Both forms of the original 1915 isolate (3326, 3326a), a grisein-producing strain of *S. griseus* (3478), and another non-streptomycin-producing culture (3482) were all insensitive to this phage, as shown by lack of multiplication or by actual adsorption by the mycelium of the organism. On the other hand, the Kelner mutant of the 1915 isolate (3326b) was phage-sensitive.

The identical behavior of the mutant of the 1915 culture with that of the streptomycin-producing strains of *S. griseus* is thus established unequivocally. Other evidence of similar cultural characteristics, sporulation, and biochemical properties further confirmed the identity of the two cultures.

These results thus suggest the probability that the 1915 isolate of *S. griseus* (3326) possessed originally the capacity of producing streptomycin, and that this capacity was lost during the more than 30 years that this culture was kept in the culture collection, growing on artificial, mostly synthetic, media. The ability of streptomycin-producing *S. griseus* cultures to mutate readily, even under ordinary conditions of culture, has been well established.[8] At least two mutants are now recognized, one of which does not sporulate and does not produce streptomycin, and one of which produces a red vegetative mycelium and forms an antibiotic substance distinct in nature from streptomycin.[4]

*Summary.* Evidence is presented that the original culture of *S. griseus* which was isolated from different soils, in 1915-1916, in the laboratories of the New Jersey Agricultural Experiment Station must have possessed the capacity for producing streptomycin. This capacity was lost upon continuous growth of

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[7] Waksman, S. A., Reilly, H. C., and Harris, D. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 617.

[8] Schatz, A., and Waksman, S. A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 129.

TABLE III.
Effect of Actinophage of Streptomycin-producing *S. griseus* on Various Strains of This Organism.

| Culture No. | Nature of strain | Spot test* for phage |  | Submerged growth effect† Phage per ml × $10^7$, after days of incubation |  |  |  |
|---|---|---|---|---|---|---|---|
|  |  | Nutrient agar | Dextrose-asparagine agar | 0 | 3 | 5 | 7 |
| 3326 | N. J. culture of 1915 isolate | — | — | 6 | 3 | 4 | 4 |
| 3326a | Holland culture of 1915 isolate | — | — | 6 | <0.4 | <0.04 | 0.004 |
| 3326b | Kelner's mutant of 1915 isolate | + | + | 6 | 100 | 105 | 186 |
| 3463 | Streptomycin-producing soil culture of 1943 | + | + | 6 | 30 | 31 | 119 |
| 3464 | Streptomycin-producing chicken throat culture of 1943 isolate | + | + | 6 | 210 | 320 | 570 |
| 3464a | Spore isolate of last | + | + | 6 | 34 | 260 | 204 |
| 3478 | Grisein-producing culture | — | — | 6 | 2 | 2 | 3 |
| 3481 | More recent independent isolate of a streptomycin-producing culture | + | + | 6 | 257 | 420 | 400 |
| 3482 | Non-streptomycin-producing culture | — | — | 6 | 0.2 | 0.01 | 0.03 |

* Phage placed on 24-hour vegetative streaks growing on nutrient and dextrose-asparagine agars, incubated an additional 24 hours, and examined for lysis.

† Phage added to freshly inoculated flasks of medium, grown under submerged conditions.

the culture upon artificial media for more than 30 years. When irradiated, this culture yielded a mutant that was as potent a producer of streptomycin as the streptomycin-yielding strains of *S. griseus* isolated in 1943.

This assumption was fully confirmed by the characteristic antibiotic spectrum of the two cultures, their behavior to streptomycin-dependent and streptomycin-resistant mutants of *E. coli* and other bacteria, and sensitivity of the two cultures to the same actinophage.

These results further point to the accuracy of the identification of the streptomycin-producing cultures of *S. griseus* with that of the original 1915 isolate, based on literature description alone, in spite of the fact that the type culture available in two collections showed distinct changes from the original description.

The authors wish to express their sincere appreciation to Dr. A. Kelner of Cold Spring Harbor for placing the mutant of *S. griseus* at their disposal, and to Mr. W. Iverson of this laboratory for assisting with some of the tests.

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## 17146. Effect of Tetraethylammonium Bromide on Adrenal Medulla.

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Acheson and Moe[1] have shown that tetraethylammonium ion will block autonomic ganglia. Since the adrenal medulla is generally accepted as ganglionic in nature, the transmission of impulses across the ganglion and subsequent release of epinephrine should be blocked by the injection of tetraethylammonium bromide. Some evidence for this has been shown by the action of tetraethylammonium on morphine hyperglycemia in dogs,[2] which closely resembles the effect on such hyperglycemia produced by surgical removal of the adrenal medulla.[3] In both cases mor-

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[1] Acheson, G. H., and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 220.

[2] Morrison, J. L., *Fed. Proc.*, 1947, **6**, 359.

[3] Bodo, R. C., Co Tui, F. W., and Benaglia, A. E., *J. Pharm. and Exp. Therap.*, 1931, **61**, 48.

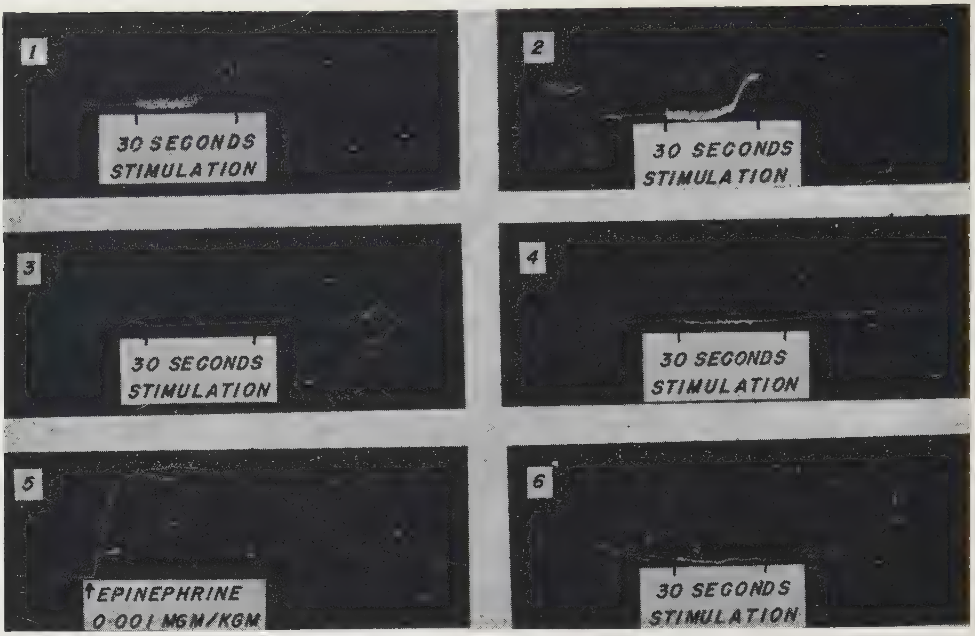

FIG. 1.

Cat C. Effect of stimulation of splanchnic nerve on retraction of nictitating membrane. (1) Initial stimulation in anesthetized cat. (2) Thirty minutes after first stimulation. (3) Ten minutes after tetraethylammonium bromide 10 mg/kg I.M. (4) Thirty minutes after injection of tetraethylammonium bromide. (5) Effect of epinephrine hydrochloride I.V. 45 minutes after tetraethylammonium bromide. (6) Seventy-five minutes after I.M. injection of tetraethylammonium bromide.

phine hyperglycemia is abolished or greatly diminished. A more direct method of determining blockade of the adrenal medulla is desirable than shown by blood sugar levels. Sensitivity of the denervated nictitating membranes of cats to circulating epinephrine offers a convenient biological test of such a blockade.[4]

*Method.* In a series of 6 cats the superior cervical ganglion on the left side was removed. During a recovery period of 4 days the nictitating membrane becomes sensitized to circulating or injected epinephrine. The cats were anesthetized and the following preparations made. Through an incision in the body wall, shielded electrodes were placed over the splanchnic nerve just before it enters the adrenal gland in order to provide for stimulation. The eyelids were retracted after cutting the lateral canthus and the nictitating membrane was connected to an isotonic lever magnifying its movement several times and recording them on a revolving smoked drum. Splanchnic nerves were stimulated using a Bird Stimulator before and after intramuscular injection of a 10% solution of tetraethylammonium bromide. In all cases after full effect of the tetraethylammonium bromide had been shown, epinephrine was injected in order to demonstrate the action of epinephrine on the nictitating membranes. Either dial solution* or pentobarbital sodium was used as the anesthetic. We could find no appreciable difference in the response of the animals to these barbiturates. Doses of tetraethylammonium bromide ranged from 3 mg/kg up to 10 mg/kg. Maximum action was obtained in 10 to 15 minutes and lasted for about an hour after which time it began to wear off. Intravenous injections of varying amounts of epinephrine hydrochloride at any time during the maximum effects of tetraethylammonium indicated that tetraethylammonium had no demonstrable effect on the action of epinephrine at the effector cells of the nictitating membrane.

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[4] Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1932, **99**, 398.

* Dial Solution furnished through courtesy of Dr. Frederick F. Yonkman, Ciba Pharmaceutical Products, Inc.

*Results.* Tracings illustrating a typical experiment in Fig. 1 show that tetraethylammonium compound blocks the release of epinephrine from the adrenal medulla.

In all of the 6 cats used in this study the nictitating membrane was stimulated to contract as a result of electrical stimulation of the splanchnic nerve. Repetition of stimuli at intervals over a period of 30 minutes to one hour produced similar contractions. Retraction usually persisted for several minutes. After administration of tetraethylammonium bromide, stimulation of the splanchnic nerve was not followed by retraction of the membrane. Injection of 3 mg/kg abolished retraction in most of the animals but 10 mg/kg abolished it in all animals. However, intravenous injection of .001 mg/kg of epinephrine hydrochloride at the peak of the tetraethylammonium effect produced marked retraction of the nictitating membrane.

*Summary.* 1. Tetraethylammonium bromide blocks the release of epinephrine from the adrenal medulla.

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## 17147. Tumorous Growths in the Pituitary and Trachea Following Radiotoxic Dosages of $I^{131}$.[*]

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The possible occurrence of thyroidal tumors after partial beta-ray destruction of the thyroid gland has prompted the investigation of long-term effects of high dosages of $I^{131}$. No thyroidal growths have been observed in our experiments with mice, but tumors of the trachea and hypophysis have been found. This report deals with the incidence of such tumors and provides a description of the growths as well as the conditions under which they are obtained. A detailed characterization of the cytological properties of the hypophyseal tumors is being prepared separately.

*Methods and materials.* Most of the work included in this report was done with adult mice of the $C_{57}$[†] strain. Smaller numbers of the A,[†] I[†] and CFW[‡] strains also were employed. Although more than 500 animals have been utilized in different phases of this study, the present report concerns 158 mice observed for a period of time sufficient to show the metaplastic and atypical growth changes, as well as 34 untreated control mice. Purina Laboratory Chow and water comprised the exclusive *ad libitum* diet of the mice, and they were kept at controlled temperature (72° ± 2°F), humidity 65% (±5%), and illumination (12 hours per day).

Radioactive iodine was given subcutaneously as NaI without carrier, in neutral aqueous solution in dosages of 10, 50, 80, 100, 160, 200, 300, 400, 500 and 1000 microcuries.[§] At least 10 animals were included in each dosage group for the $C_{57}$ and CFW strains, and in most cases, especially with the 100, 200 and 300 microcurie dosages, there were 40 or more animals.

Calibration and measurement of the dosages was done with a Geiger counter and standards

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[*] This study has been supported in part by the National Cancer Institute, U. S. Public Health Service, and in part through contract with the Atomic Energy Commission and Office of Naval Research.

[†] Breeding stock of these strains originally obtained from colony of L. C. Strong, Yale University.

[‡] Purchased from Carworth Farms, New City, N. Y.

[§] 100 $\mu c$ in normal adults produces almost complete, and 200 $\mu c$ complete, thyroidal destruction.[1]

[1] Gorbman, A., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 212.

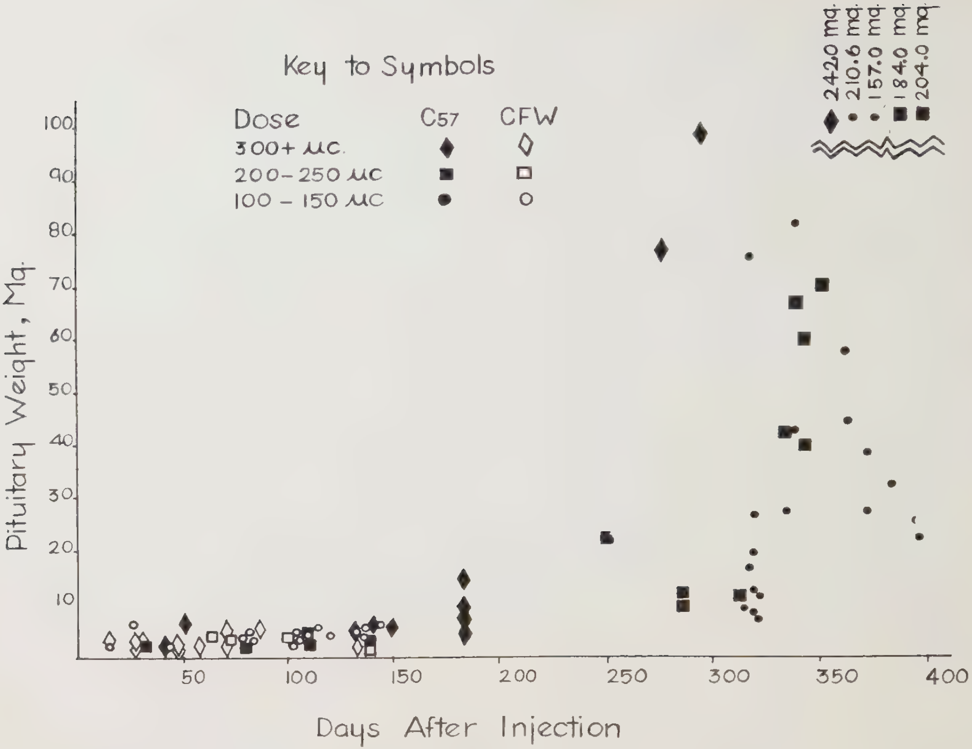

FIG. 1.
Weights of pituitary glands after injection of $I^{131}$.

which consistently have given a millicurie unit with about 90% of the activity indicated by measurements of the Oak Ridge National Laboratory. Measurement of $I^{131}$ uptake in several separate series of mice injected under these conditions showed that only 5% to 10% of the administered $I^{131}$ is stored in the thyroid, reaching this maximum 12 to 14 hours after injection. The pituitary in our observations never contained $I^{131}$ in concentrations significantly higher than the blood.

Animals were sacrificed in groups at intervals, or singly at irregular times if moribund. Pituitaries were fixed in 10% formalin and weighed from 70% alcohol. Tracheas were sectioned serially for microscopic examination.

*Observations.* The 10 μc and 50μc dosages were without effect upon the pituitary, as judged by weight. The 80 μc dose was similarly ineffective in producing tumorous pituitaries, although it did elicit a 3-fold or 4-fold enlargement of the gland in animals which were of small weight at the time of injection. The 80 μc dose also produced metaplastic changes in the tracheal epithelium. Dosages of 400 μc or more almost always resulted in death at an interval too early for development of hypophyseal tumors. For these reasons detailed discussion of results is confined to animals given less than 400 μc of $I^{131}$.

*Pituitary gland.* Fig. 1 indicates the general pattern of results obtained. It seems clear that soon after injection of 100 μc to 325 μc quantities of radioactive iodide, and up to 150 days thereafter, there was mild enlargement of the hypophysis. The average control pituitary weight in this interval was 1.2 mg. The average of $I^{131}$-injected animals was 3.34 mg. Between 150 and 250 days rapid increments in pituitary weights occurred in mice given adequate $I^{131}$ dosage. As indicated in Fig. 1, after 250 days very few pituitaries under 10 mg could be found. The average control pituitary of older mice weighed 2.1 mg. The average of all the $I^{131}$ mice was 34.7 mg. The data indicate a tendency for larger tumors of earlier occurrence after the largest (300+ μc) dose. The 3 largest tumors weighed 242, 204 and 210 mg, and followed 325 and 200 and 100 μc dosages, respectively. Excluding 5 extremely large glands of over 150 mg, the average weights of pituitaries 250 days or more following injection, were 27.5 mg for the 100-160 μc dosage group, 39.7 mg for the 220-225 μc group, and 87.3 mg for the 300+ μc group.

The glands weighing more than 20 mg characteristically showed hemorrhagic areas, particularly on the dorsal side near the infundibular stalk. Gross examination of serial sections of the tumors proved that they are limited to the anterior lobe.

Pituitaries weighing more than 30 mg frequently produced blindness and motor disturbances in the mice. Such glands lost the smooth outlines of smaller tumors and frequently were lobed. In most instances a hollow was formed on the ventral side of the brain by displacement of its parts. The larger tumors, by pressure against the brain, actually caused various degrees of separation of the parietal and occipital bones. The resulting distortion in the shape of the head made it possible to identify, from external examination, those animals with tumorous pituitaries. All of the hypophyseal tumors appeared to exert their influence upon neighboring tissue by pressure alone. In no instance was there evidence of erosion or invasion of brain or bone or other tissue by growing hypophyseal masses.

*Trachea.* In contrast with most other tissues, the tracheal epithelium appeared extremely sensitive to the accumulated radiant energy in the thyroid gland, and cellular responses were visible as early as one day following $I^{131}$ injection. Early responses were all of the degenerative type, and their degree and rate of development varied with the size of the $I^{131}$ dose.

Pycnosis of nuclei, loss of cilia, and finally death and desquamation of the cells was the usual order of changes observed after dosages of 80 μc or more. Even with the lowest effective dose such changes were completed within a week, and regeneration begun at that interval. The 80 μc dose produced minimal lesions; the 1000 μc dose produced within 48 hours complete desquamation of that tracheal epithelium closest to the thyroid. In cases of large scale loss of the tracheal epithelium accumulations of macrophages appeared within several days and were seen to contain cellular debris. After the 80-160 μc doses, tracheal epithelial regeneration was, as a rule, of the normal type and occurred from the normal epithelium surrounding the lesions. After doses of more than 200 μc tracheal regeneration was, as a rule, with a metaplastic epithelium of variable form and only rarely entirely normal. Sometimes it consisted of cuboidal ciliated or unciliated cells. Most often, especially with the higher doses, it was simple or stratified squamous in type. Some of the stratified squamous tracheal epithelia in CFW mice were extremely active mitotically.

Changes in the fibrous tunica propria and in the tracheal cartilages also occurred, but only with doses in excess of 300 μc. Temporary loss of basophilia in the cartilage often was observed soon after injection, and death of many chondrocytes was usual. Such changes often were followed by a hyaline thickening of the perichondrium which in some instances became as thick as the entire cartilage and in some instances strongly resembled bone. Thickening of the fibrous tracheal tunica propria was usual after the higher doses in animals sacrificed about six to ten months after injection. In 5 extreme cases the fibrous thickenings projected into the tracheal lumen as small tumors almost completely obstructing it. Such animals before autopsy had a labored wheezing type of breathing.

*Discussion.* The mechanism by which tumorous growth in pituitaries was elicited in our experiments does not seem to be deducible from the data at our disposal. Administration of goitrogens leads to mild enlargement of the pituitary gland.[2] In this laboratory (unpublished data) a 0.1% thiouracil diet fed to mice for almost 400 days yielded pituitary weights as high as 8.2 mg, and averaging 4.4 mg, compared to the control weight in old mice of 2.0 mg.

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[2] Higgins, G. M., *Am. J. Med. Sci.*, 1945, **210**, 347.

It does not seem likely, therefore, that the radiation thyroidectomy experienced by our animals is the principal factor to be considered, though it conceivably might play a synergistic role in combination with some other influence.

It has been well established[3,4] that chronic administration of estrogens to rodents results in tumorous enlargements of the pituitary. It is tempting to note that many of our long-term $C^{57}$ females were in extended periods of vaginal cornification, and had enlarged mammary glands and uteri, as well as loosening of the pubic symphysis, when sacrificed 300 or more days after treatment. On the other hand, male $C_{57}$ mice were found to have some of the largest hypophyseal tumors in the series and yet displayed none of the usual signs of estrogenization. At the present time, attempts are being made at experimental analysis of this situation, but a really rational approach to the problem does not seem to be indicated by the available data.

It would seem that the destruction of tracheal epithelium in our mice is a direct result of the ionizing beta radiation emanating from the thyroid gland. The regenerated epithelia, though usually metaplastic never showed neoplastic tendencies, excepting possibly those cases of stratified squamous epithelia which were mitotically extremely active. Though apparently benign, the tracheal tumors found in our mice were, or were about to be, fatal through tracheal obstruction and hindrance to respiration.

It should be emphasized that the range of beta particles of $I^{131}$ in tissue is usually considered to be approximately 2 mm.[5] The thyroid capsule alone is as thick as this around most human thyroids, and hence beta irradiation of thyroid-contiguous tracheal structures appears unlikely. Nevertheless, attention has been drawn[6] to the relatively frequent occurrence of signs of tracheal injury in patients undergoing $I^{131}$ therapy for hyperthyroidism. The possibility of fibrous tracheal proliferation in humans following $I^{131}$ treatment of the thyroid remains to be considered.

*Summary.* Following doses of radioactive iodine sufficient to cause destruction of most or all of the thyroid, tumorous enlargements were found in the pituitary glands and tracheas of mice. Hypophyseal growths as large as 240 milligrams (control, 2.1 mg) were found. Less than 10% of animals sacrificed more than 250 days after $I^{131}$ administration had pituitaries smaller than 10 mg in weight.

Tracheal tumors of the fibrous tunica propria, obstructing the tracheal lumen, were of lower incidence (4%) but appeared earlier. Fibrous thickenings of the tunica propria, and metaplastic regeneration of tracheal epithelium were more common.

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[4] Wolfe, J. M., and Wright, A. W., *Endocrinology*, 1938, **23**, 200.

[5] Marinelli, L. F., Quimby, E. H., and Hine, G. J., *Am. J. Roentgenol. and Rad. Therap.*, 1948, **59**, 260.

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## 17148. Influence of Environment on Preweaning Growth of the Rat. I. Dietary Regimen of the Young.*

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Evidence presented in a previous publication[2] indicated that the growth curve of preweanling rats in the authors' laboratory did not conform to the formulation of preweaning growth given by Zucker *et al.*[3,4] It was found with the Anderson and Smith[5] diet that Zucker's equation, $\log W = k \log t + \log C$, applied to the approximate postconception age periods, 22 to 34 and 43 to 50 days, but that there were changes in the constants of this equation at fairly definite ages. The first change at approximately 24 days was believed to be correlated with the birth-weight but the second change at about 29 days of age was tentatively ascribed to a change in growth pattern. The divergence of the curve from a straight line at about 34 to 43 days was assumed to be due primarily to some dietary influence.

These results were obtained under the usual breeding conditions wherein the mother and young were housed in a single cage and all had access to the diet. It is generally recognized that the young begin to ingest solid food coincident with the opening of their eyes at about 34 days after conception and it has been suggested[2] that this phenomenon may be associated with the divergence of the growth curve. The following experiments, fashioned after those of Kozlowska *et al.*[6] except that no mineral supplements were employed, were performed to elucidate the factors contributing to the observed divergence and to determine the pattern of growth when the young are restricted to the mother's milk.

*Experimental.* Seven pregnant Long-Evans strain rats were placed in individual breeding cages. The first group (3 rats) was fed the Anderson and Smith diet *ad libitum* in the breeding cages until the young were weaned. The second group (3 rats) was removed from the breeding cages (which contained no food) for 2-hour periods 3 to 4 times daily during the period 7:30 A.M. to 10:30 P.M. and allowed to eat the same diet *ad libitum* in separate cages. This procedure was continued until the young were weaned at 50 days postconception (28 days, post-natal). At this point the mother was removed from the breeding cage and the young were given the Anderson and Smith diet *ad libitum*. The third group (1 rat) was fed similarly except that separate feeding of the mother was discontinued at 43 days postconception and the diet was made available *ad libitum* in the breeding cage until the young were weaned at 50 days postconception.

Each of the litters was reduced at birth to 6 young divided, so far as possible, equally between males and females. The total weight of each sex in each group was determined daily and the total weights were divided by the number of males and females, respectively. The plot of the resultant averages is shown in the accompanying figures. These procedures afforded growth data on a total of 39 young (18, 15 and 6, respectively) in the 3 groups.

*Results.* The growth curves obtained for the male rats are shown in Fig. 1. Only the curves for the males are given inasmuch as the results were similar with the females. Since the growth data of the 10 males of

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* Paper 60. For Paper 59 see Dunn *et al.*[1] The writers' work has been aided by grants from the Nutrition Foundation, Inc., and the University of California. The authors are indebted to Walter S. Trasin for technical assistance.

[1] Dunn, M. S., Camien, M. N., Eiduson, S., and Malin, R. B., in press.

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[3] Zucker, L., Hall, L., Young, M., and Zucker, T. F., *Growth*, 1941, **5**, 399.

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[5] Anderson, W. E., and Smith, A. H., *Am. J. Physiol.*, 1932, **100**, 511.

[6] Kozlowska, C. M., McCay, C. M., and Maynard, L. A., *J. Nutr.*, 1932, **5**, 61.

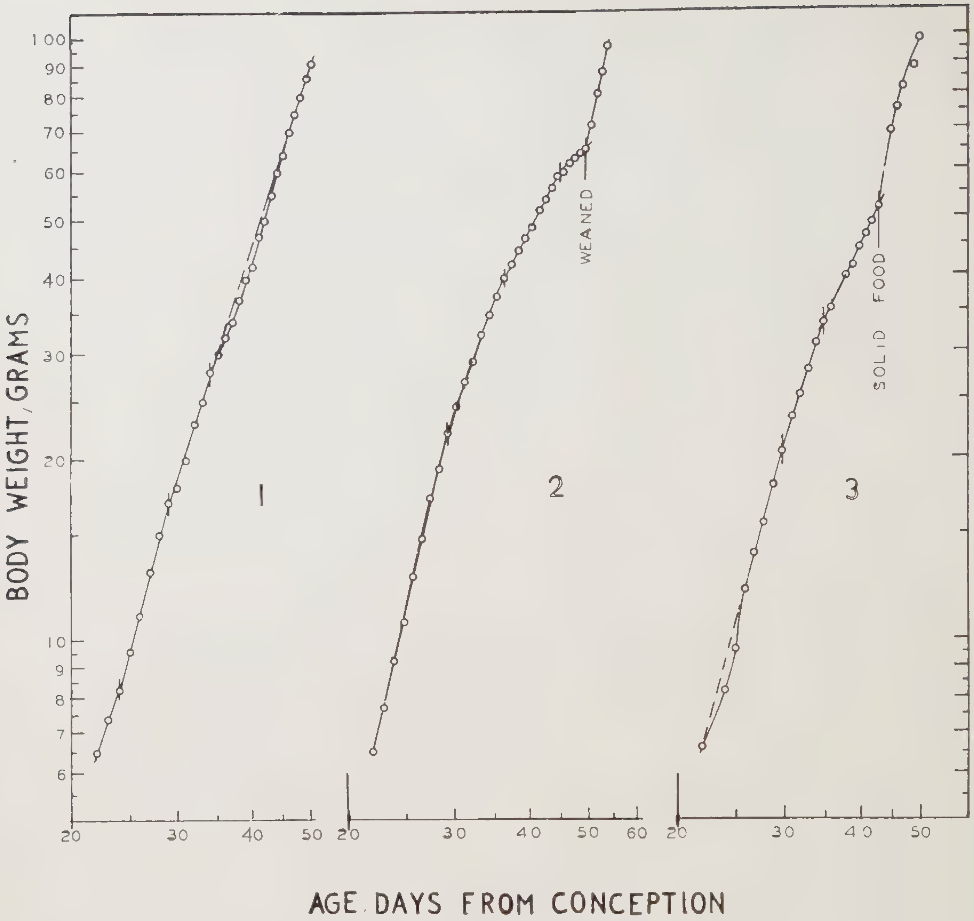

FIG. 1.

Logarithmic curves relating body weight and age of male Long-Evans strain rats under different dietary conditions.

Curve 1. Average of 33 rats, unrestricted feeding.
Curve 2. Average of 10 rats, restricted until weaning.
Curve 3. Average of 4 rats, restricted until 43 days of age.

Group 1 and those obtained previously were in close agreement the two sets of data were combined. The resultant curve (Curve 1, Fig. 1), therefore, represents the average growth of 33 males. The average curves representing the nine males of Group 2 and the 4 males of Group 3 are shown as Curves 2 and 3 of Fig. 1, respectively.

*Discussion.* It is apparent that the shape of the growth curve of the young, particularly from about 34 days of age (postconception) to weaning, is profoundly affected by the availability of solid food. The changes in slope of Curves 2 and 3 occurring between 34 days and weaning may reflect both the decreasing food value to the young rat and the decreasing volume of the mother's milk.[2] The insufficiency of the milk as a food source with increasing size of the young rat is emphasized by the abrupt increase in growth rate as soon as solid food became available. The correlation of these data is shown in Fig. 2 which was constructed by superimposing the curves for the male rats given in Fig. 1. This was accomplished by aligning the tracings so that point B and the line BC on each of the 3 curves coincided. In this way the curves are plotted approximately identically on the X scale (age) without regard for the Y scale (weight). The effect of environment on abso-

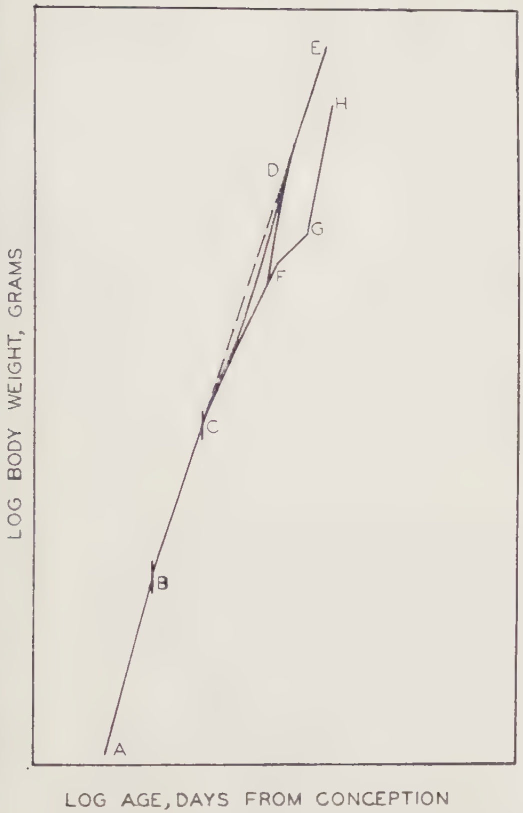

Fig. 2.

Superimposed logarithmic curves relating body weight and age of male Long-Evans strain rats under different dietary conditions.

Solid line, ABCDE—Curve 1 of Fig. 1. Unrestricted feeding.

Solid line, ABCFGH—Curve 2 of Fig. 1. Restricted, to 50 days.

Solid line, ABCFDE—Curve 3 of Fig. 1. Restricted, to 43 days.

lute size will be considered in a subsequent publication. In Fig. 2 the solid line, ABCDE, represents the growth of the males in Group 1 (unrestricted feeding); line ABCFDE represents the growth of the males in Group 3 (restricted to 43 days); and line ABCFGH represents the growth of the males in Group 2 (restricted to 50 days, weaning). It may be concluded from these results that the slopes of the segments CF and FG represent the rates of growth natural to the milk diet during these age periods and that the slope of segment DE is typical of the natural rate of growth on the solid diet after 43 days of age. The shape of the growth curve of the unrestricted males, therefore, would appear to be the resultant of the natural transition from the milk diet (CF) to that of the completely solid diet (DE) with the completion of the physiological ability to utilize fully the solid diet occurring at about 43 days (postconception). In this case the curve is naturally concave. Since, in the Group 3 animals there is present the physiological capacity to utilize the solid food, the resultant convexity of the curve is indicative of the "recovery" nature of the growth. This reasoning also accounts for the increase in slope of GH over that of DE.

Since the data of Group 1 agree very well with other similar data obtained earlier and with different groups of animals, they have reasonable reliability. That segments of the 3 curves have the same relative shape and are superimposable is further evidence supporting this view. It would seem to be a logical conclusion that the divergence from a straight line between about 34 to 43 days (illustrated by Curve 1, Fig. 1 and reported previously[2]) does not indicate necessarily a qualitative deficiency in the diet but may be a natural phenomenon arising because of the transition from a milk diet to one of solid food. Although this explanation was suggested by MacDowell *et al.*,[7] the present experiments appear to be the first to demonstrate its validity. That the causes were quantitative, rather than qualitative, in nature was indicated in previous experiments[2] with a limited amount of data. It was also shown earlier that the degree of divergence and/or its duration could be influenced by the quality of the diet.

*Summary.* The shape of the preweaning growth curve of the rat is profoundly affected by the lack or availability of solid food. The divergence from conformity to Zucker's growth equation of rats raised on the Anderson and Smith diet may be due to the natural transition from a liquid (milk) to a solid diet and does not appear to be explained by a qualitative deficiency in this diet.

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## 17149. Electrical Precipitation of Egg-White Inhibitor of Influenza Virus Hemagglutination.*

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During electrophoresis of solutions of crude egg-white or semi-purified egg-white inhibitor[1] of virus hemagglutination, there frequently develops a voluminous, gelatinous precipitate which contains considerable inhibitory activity. This phenomenon and its possible significance are discussed in the present report.

*Materials and methods.* Egg-white (EW) was collected from 1-day-old hen's eggs. The thick white was obtained apart from outer and inner thin,[2] diluted 4-fold, and homogenized *lightly* in a Waring blendor. Semi-purified inhibitor was prepared from whole or thick EW by precipitation with 7 volumes of 0.1 M $KH_2PO_4$ and extraction of the precipitate, after washing, with 0.06 M phosphate buffer of pH 7.2[1]. Electrophoresis was carried out in the Tiselius cell with dialyzed solutions in veronal buffer at pH 8.6 or phos-

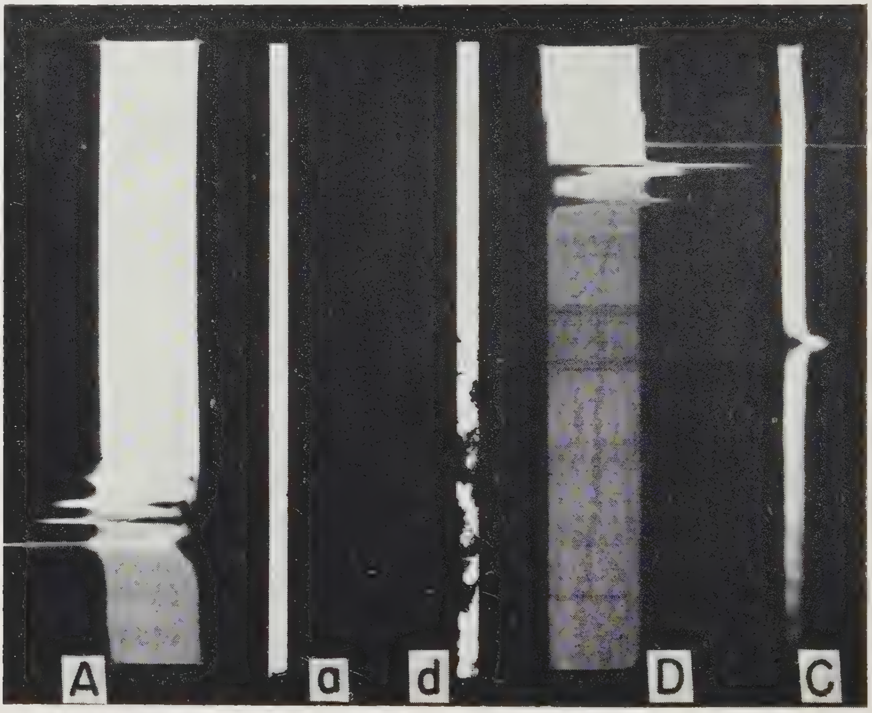

Fig. 1.
Photograph of ascending (a) and descending (d) limbs of Tiselius cell after 3 hr showing precipitate in (d). Diagrams of boundaries and the opacity due to precipitate in ascending (A) and descending (D) limbs after 22 min. The diagram of the slowest component after 215 min. is shown in C.

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* This investigation was supported by a research grant from the National Cancer Institute, U. S. Public Health Service; by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., and by the Dorothy Beard Research Fund.

[1] Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, in press.

[2] Hughes, J. S., and Scott, H. M., *J. Poultry Sci.*, 1936, **15**, 349.

TABLE I.
Properties of Fractions Obtained by Electrophoresis of EW Inhibitor Preparation A200 PII EII in Phosphate Buffer of 0.1 Ionic Strength and pH 7.2.

| Fraction | Vol. recovered (ml) | N ($\gamma$ per ml) | Inhibition titer | Partition of total recovered, N (%) | Partition of total recovered, activity (%) | Purification factor* |
|---|---|---|---|---|---|---|
| Starting material | | 95.5 | 12,800 | | | 40 |
| Descending limb | 6.1 | 25.8 | 1,500 | 19 | 6.8 | 17 |
| Ascending ” | 6.4 | 17.2 | 2,700 | 13 | 13 | 47 |
| Bottom cap | | | | | | |
| (a) supernatant | 2.2 | 85.6 | 12,100 | 22 | 20 | 42 |
| (b) precipitate† | 1.3 | 298 | 62,000 | 46 | 60 | 62 |
| (c) total‡ | 3.5 | 165 | 31,000 | 68 | 80 | 56 |
| Crude EW | | 16,700 | 55,500 | | | 1.00 |

* Calculated from the titer/N ratio with reference to the titer/N ratio of crude EW.

† An almost transparent gel. Deducting the nitrogen and inhibitory activity of an equal volume of supernatant, one obtains the value 71 for the purification factor of the solid phase.

‡ Calculated by combining the results for supernatant and precipitate.

phate buffer at pH 7.2, each at 0.1 ionic strength.† Inhibitory activity was titrated against 4 hemagglutinating doses of heated (53°C, 30 minutes) purified swine influenza virus.[1] Nitrogen was determined by direct nesslerization.

*Experimental.* The following experiment is illustrative. Semi-purified inhibitor, dialyzed against phospate buffer, was submitted to electrophoresis at 5 volts per cm. Within 3 minutes, the whole solution, initially almost clear, acquired a ground-glass appearance. Floccules formed gradually and settled toward the bottom cap. Precipitate remaining in the descending limb after 3 hours is shown in Fig. 1d; settling was often complete in the ascending limb (Fig. 1a). The isolated contents of the 2 limbs and bottom cap, the latter after centrifugation for 10 minutes at 870 g, were analyzed for nitrogen and inhibitory activity. The results (Table I) show (a) concentration of inhibitory activity in the bottom cap; (b) association of the activity with the precipitate; and (c) purification of the inhibitor consequent to electrical precipitation.

Precipitation occurred frequently during electrophoresis of solutions of whole or thick EW, with results analogous to those of Table I. The precipitation seemed more pronounced in veronal than in phosphate buffer and could be prevented in the former by vigorous blending without significant decrease in inhibitory activity. With semi-purified inhibitor, vigorous blending delayed the onset of precipitation, making it possible to record boundaries, as shown in Fig. 1 (A and D). In the absence of precipitation, neither inhibitory activity nor nitrogen was concentrated in the bottom cap. In contrast with precipitates obtained chemically,[1] these precipitates dissolved readily in dilute phosphate buffer. Indeed, resolution occurred in the cell if the current was discontinued shortly after the onset of precipitation.

During the initial period of precipitation in thick EW, the precipitate moved in the cell like a protein component too dilute to register as a distinct peak of refractive index gradient but visible because of its light-scattering capacity. The diffuse edges of this material were visible, one in each limb, moving a little slower than the ovalbumin peaks. The peaks located in the region of precipitation were initially obscured, but reappeared as the precipitate settled, and the final diagram did not differ significantly from diagrams of materials in which no precipitation occurred. With purified inhibitor, the precipitate moved well ahead of the slow component.[1]

*Discussion.* While the mechanism of precipitation during electrophoresis is not yet clear, the electric field, pH, and inhibitor concentration may be recognized as contributing factors. Moreover, the blending experiments indicate that the physical properties of the inhibitor in solution are important for the phenomenon and that it is possible to modify some of these properties without appreciably affecting biological activity. Solutions of purified inhibitor have a high viscosity, suggesting the presence of a highly asymmetric component.[1,3] Although it has not been possible to observe birefringence during electrophoresis of these solutions, it is possible that predisposition to aggregation results from orientation of asymmetric particles in the electric field, as in the Kerr effect.[4] The application of the electrical precipitation phenomenon to the final purification of the EW inhibitor is being explored.

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† Precipitate containing inhibitory activity frequently developed during dialysis of 25% whole or thick EW and was removed by centrifugation prior to electrophoresis.

*Summary.* Precipitation occurs in solutions of egg-white or purified egg-white inhibitor of virus hemagglutination submitted to an electric field. The precipitate includes a great part of the inhibitor of the preparations in a state substantially purer than that of the starting material. This result suggests application of the phenomenon to the final purification of the inhibitor.

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## 17150. Propagation of Canine Distemper Virus on the Chorio-Allantoic Membrane of Embryonated Hen Eggs.

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Several attempts have been made by various investigators to cultivate canine distemper virus in the tissues of developing chick embryos. Most of these attempts were not too successful. Thus, Mitscherlich[1] found the virus to survive for 6 days after inoculation onto the chorio-allantoic membrane, but not to be active after subinoculation to other eggs. Plummer[2] in two attempts maintained the virus for 5 and 6 passages respectively on the chorio-allantois but failed to carry it further. Beveridge and Burnet[3] obtained no evidence that canine distemper virus multiplied in chick embryo tissues although various routes of inoculation were tried.

However, Haig[4] reported the successful cultivation of Green's distemperoid virus through 30 serial passages on the chorio-allantoic membrane of chick embryos, and later reported[5] that the virus had been maintained on the chorio-allantoic membrane for more than 90 serial passages over a period of 15 months. During this time the egg-adapted virus was found to have become partially attenuated for ferrets and to have lost some of its highly contagious characteristics for this species. A temperature of 37°C seemed to be the most suitable for incubation of the inoculated eggs since both higher virus titers and more pronounced macroscopic lesions on the chorio-allantoic membranes were attained. Attempts to maintain the virus by serial passage by the yolk-sac, allantoic cavity or amniotic cavity routes of inoculation ended in failure.

For some time attempts have been made in this laboratory to adapt canine distemper virus to some host other than dogs or ferrets. In accordance with the experience of most previous investigators, most of our attempts ended in failure. However, in studies initiated before the work of Haig[4,5] appeared in print, we too obtained results indicating that canine distemper virus may be successfully passed for a number of generations on the chorio-allantoic membrane of developing chick embryos. The object of this paper is to present our experimental data.

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[1] Mitscherlich, E., *Dtsch. tierarztl. Wschr.*, 1938, **46**, 497.

[2] Plummer, P. J. G., *Canad. J. Comp. Med.*, 1939, **3**, 96.

[3] Beveridge, W. I. B., and Burnet, F. M., Med. Res. Council, Sp. Report Series, No. 256, 1946, p. 76.

[4] Haig, D. A., *Onderstepoort J. Vet. Sci. and Animal Industry*, 1948, **23**, 149.

[5] Haig, D. A., *J. South Afr. Vet. Med. Assn.*, 1948, **19**, 73.

*Material and methods.* The distemper strain was the stock virus used by the Lederle Laboratories* for the production of canine distemper vaccine and immune serum. This strain of virus, hereafter referred to as the *Lederle* strain, was isolated from a sick dog in these laboratories in 1941 and has been maintained since then by irregular alternate passages through ferrets and dogs. When inoculated into ferrets, this strain produces the usual symptoms and lesions characteristic of distemper: pyrexia, general erythema with rash around the mouth, which usually becomes pustular; erythema and edema of the anus; inflammation of the nasal passages and mucous membranes of the eyes, which is shown first by a watery discharge that later becomes purulent, causing edema and glueing of the eyelids. All inoculated ferrets show these symptoms 6 to 10 days following inoculation and usually die 2 to 3 days later.

The ferrets used in the experiments were usually between 3 and 10 months old. They were bred on an upstate New York farm and when brought to the laboratories were kept in strict isolation at an adequate distance from the infected area. They were cared for by attendants who never came in contact with dogs or the infected ferrets.

Seven-day-old developing chick embryos were used throughout. Inoculations were made either onto the chorio-allantoic (C-A) membrane or into the yolk sac. The inoculated eggs were incubated either at 35° or 38°C for 4 to 5 days at which time the chorio-allantoic membranes and/or yolk sacs, embryos and allantoic fluids were harvested under aseptic conditions. All suspensions were made in beef heart infusion broth free of dextrose.

*Experimental.* Of the various routes and series of inoculations made, the series that was followed with best success is described in detail: A 20% suspension of infected ferret spleen was centrifuged at 2,000 r.p.m. for 10 minutes. To the supernate penicillin and streptomycin was added to give 500 units and 100 $\mu$g of each respectively per ml and after standing for one hour at room temperature, 12 eggs were inoculated with 0.2 ml each on the C-A membranes and then incubated at 35°C. One embryo was dead on the first day and was discarded. The remaining 11 embryos were still alive at the end of the 5th day. The C-A membranes appeared normal. They were harvested and a 20% suspension of their pooled tissue, after treatment with penicillin and streptomycin as described above, was inoculated on the C-A membranes of a second series of 15 eggs. A total of 4 serial passages in eggs was thus made. C-A membranes of the 4th passage were pooled and 0.5 ml of a 20% suspension of the pool was injected subcutaneously into each of 2 ferrets. Both ferrets developed symptoms typical of distemper 9 and 10 days later, respectively, and were found dead on the 12th day.

A 20% spleen suspension of one of the ferrets was inoculated into 10 developing chick embryos by the yolk-sac route. Four days later the yolk sacs of 8 surviving embryos were harvested and a portion of the pooled tissue suspension subinoculated onto the C-A membranes of 10 eggs. Two more serial passages were made on the C-A membranes and then pooled membranes of the last passage were made up to a 20% suspension and injected subcutaneously in 0.5 ml amounts into each of 2 ferrets. Both ferrets showed symptoms indicative of distemper on the 7th day and were found dead on the 9th day. A suspension of their pooled spleens was prepared and a portion inoculated onto the C-A membranes of 14 eggs. These eggs constituted the first passage of our current strain in eggs which now has been carried for 74 successive serial transfers on the C-A membranes.

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* The authors are indebted to Mr. H. M. Kroll, Animal Industry Section, Lederle Laboratories, for his cooperation in the initial stages of the work.

Our experience with the *Lederle* strain propagated on the C-A membranes has been very similar in many ways to that reported by Haig[4,5] for the *Onderstepoort* strain. Up through the 9th to 10th passage of the *Lederle* strain very little, if any, changes were noticed in the appearance of the C-A membranes. However, starting at that passage level some of the membranes were noted to be thickened and moist, showing fairly numerous small areas whitish-gray in appearance. These changes became more marked with increasing number of egg passages so that to date, at the 74th passage, the membranes are markedly thickened and the accompanying lesions quite numerous, heavy and opaque (see Fig. 1).

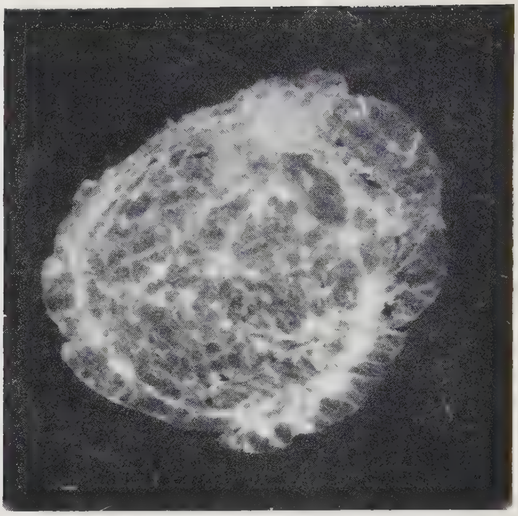

FIG. 1.
Distemper infected chorio-allantoic membrane.

Appropriate bacteriological tests carried out routinely with both aerobic and anaerobic media have consistently shown the infected tissues to be free of any detectable bacterial contaminants.

*Subinoculation of the egg-adapted virus into ferrets.* At intervals membranes of a certain passage level were pooled, homogenized to a uniform suspension and inoculated into ferrets for testing of virulence. The data pertaining to the maintenance of the virus in eggs and subinoculation into ferrets are summarized in Fig. 2.

In brief, it was found that canine distemper virus may be maintained by serial passage on the C-A membranes of developing chick embryos and that the virus still retains its

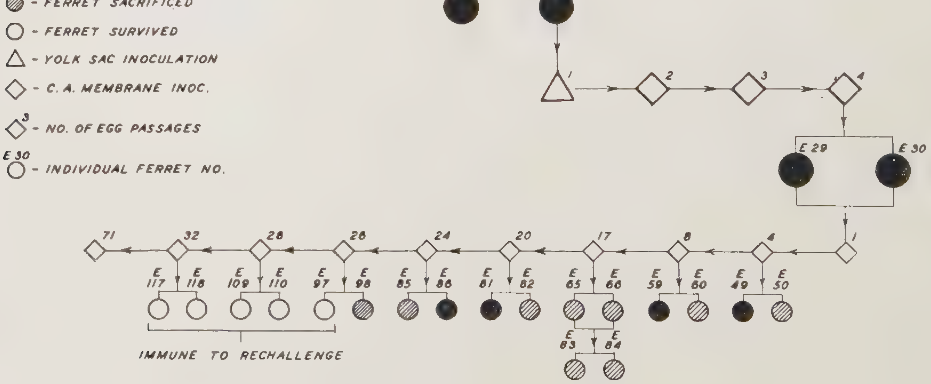

FIG. 2.
Ferret and egg passage series of distemper virus.

lethal effect for ferrets through at least the 20th egg passage. Starting at the 26th passage level, however, the C-A membrane material was definitely of lessened virulence since 1 of 2 ferrets survived infection following a very mild illness and was found to be completely resistant on rechallenge with approximately 100 MLD of virulent ferret spleen virus 30 days later. Likewise ferrets inoculated with C-A membranes of the 28th and 32nd egg passages showed no signs of infection although they too were found completely resistant to rechallenge with approximately 100 MLD of virulent ferret spleen virus 30 days later.

*Further proof for identity of the virus.* In order to eliminate the possibility of having viruses other than that of canine distemper present, the following animals were inoculated with a 20% suspension of C-A membranes of the 48th egg passage: 6 Swiss albino mice with 0.03 ml each intracerebrally; 3 guinea pigs with 0.15 ml each intracerebrally; 3 guinea pigs with 1.0 ml each subcutaneously; 2 rabbits on the scarified corneas with 0.1 ml each, and 2 ferrets with 1.0 ml each intraperitoneally. None of the inoculated animals showed any signs of illness, therefore infection with other viruses such as lymphocytic choriomeningitis or herpes simplex was improbable. The 2 ferrets were challenged at the end of 30 days and were shown to be immune to at least 100 MLD of virulent ferret spleen distemper virus.

*Neutralization tests in eggs.* Dogs, ferrets and guinea pigs were hyperimmunized with repeated injections of ferret spleen virus. Blood samples were drawn and the sera from each species pooled. Pools of normal ferret serum and normal guinea pig serum were also prepared. The 5 serum pools were filtered separately through single Seitz EK pads and inactivated at 56°C for 30 minutes. Each serum pool was then mixed with an equal volume of a 20% suspension of C-A membranes of the 55th egg passage. As a further control a mixture of equal volumes of sterile broth and C-A membrane suspension was similarly prepared. All 6 mixtures were incubated at 37°C for 2 hours before inoculation into 6 eggs each on the C-A membranes. All eggs were opened after 5 days' incubation at 35°C and the embryonic membranes and tissues carefully examined macroscopically. The results may be summarized by stating that those eggs inoculated with the mixtures containing normal ferret serum, normal guinea pig serum or sterile broth all showed the markedly thickened membranes and numerous opaque foci that are usually observed with the *Lederle* strain of egg-adapted canine distemper virus. In contrast those eggs inoculated with the mixtures containing immune guinea pig, ferret or dog sera showed only the typically thin, sparkling transparent appearance of normal membranes with no visible lesions whatsoever.

*Neutralization tests in ferrets.* Two ferrets were hyperimmunized by injecting them subcutaneously at 5-day intervals with 1 ml of a 20% C-A membrane suspension representing the 59th, 60th, 61st, 62nd, 63rd and 64th egg passages. The ferrets were bled by cardiac puncture one week after the last injection and their sera pooled. Three guinea pigs which were hyperimmunized in a similar manner were likewise bled and their sera pooled. In addition 2 other ferrets were submitted to the same schedule of immunization with the exception that they received an additional intraperitoneal injection of 2.0 ml of a 20% suspension of the 65th C-A membrane passage. They were also bled one week after the last injection and their sera pooled. Pools of normal ferret and of normal guinea pig sera were also prepared. The 5 serum pools were filtered separately through single Seitz EK pads and inactiviated at 56°C for 30 minutes. Mixtures, containing 8 ml of the respective serum plus 2 ml of infected ferret spleen suspension (containing at least 100 MLD of virulent virus per ml) were incubated at 37°C for 2 hours and then injected intraperitoneally into 2 ferrets, each ferret receiving 5 ml of the serum-virus mixture. The results may be summarized by stating that those ferrets inoculated with the mixtures containing normal ferret or guinea pig serum showed typical symptoms of distemper starting either on the 7th or 8th day and were dead by the 11th to 13th day. In contrast the 6 ferrets inoculated with the mixtures containing immune ferret or guinea pig sera remained symptom-free and survived in a healthy state for more than 35 days following inoculation.

From the two neutralization tests described above it is apparent that both the egg-adapted virus strain and the parent strain of ferret spleen virus are antigenically (immunologically) related.

*Comment.* During the course of the work described above, Haig[4,5] reported his studies in which he successfully maintained the *Onderstepoort* strain of virus for more than 90 serial passages on the C-A membranes of chick embryos over a period of 15 months. Through the courtesy of Dr. D. A. Haig and Dr. R. Alexander,[6] Director of the Division of Veterinary Services, The Veterinary Research Institute, Pretoria, Onderstepoort, Union of South Africa, the *Onderstepoort* strain of virus was sent to us in December 1948 and was readily established and maintained in our laboratory on the C-A membranes of chick embryos. Studies concerning the comparative properties of the *Onderstepoort* and *Lederle* strains of chick embryo adapted distemper virus will be published at a later date.

*Summary.* Studies are reported in which the *Lederle* stock strain of canine distemper virus was adapted and maintained through 74 serial passages on the chorio-allantoic membranes of developing chick embryos. The chick embryo adapted strain retained its capacity to produce lethal infection in ferrets through the 24th egg passage level but apparently lost its ability to do so between the 24th and 28th egg passages. Ferrets inoculated with chick embryo adapted virus of passage levels higher than the 28th transfer failed to show any signs of disease, but were found immune on rechallenge with fully virulent ferret spleen virus. Cross neutralization tests carried out in chick embryos and in ferrets indicate that the chick embryo adapted strain and the parent strain of ferret spleen virus are immunologically related.

The authors wish to express their appreciation to Mr. Frank E. Smith for his able technical assistance during the course of these studies.

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[6] Alexander, R., personal communication to H. R. Cox.

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## 17151. Chymotrypsin in Cancer.

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A trial of chymotrypsin as a therapeutic agent in cancer was initiated at the Laboratory of Experimental Oncology in August 1948. The isolation of this enzyme in the crystalline state was described by Kunitz and Northrop.[1] The basis of interest in chymotrypsin in cancer was not the highly tenuous theoretical considerations that apparently led to its use,[2] but the purported clinical results in one case that has appeared in the literature[3] and additional similar cases that were rumored in this area and elsewhere.[4] These reports claimed prompt relief of pain after 3 or 4 parenteral injections of chymotrypsin, and subsequent decrease or disappearance of lesions stated to have been malignant neoplasms.

Ten patients (Table I) with inoperable neo-

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* Aided by Grant 396 from the National Advisory Cancer Council.

[1] Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1935, **18**, 433.

[2] Gurchot, C., Krebs, E. T., Jr., and Krebs, E. T., *Surg., Gynec. and Obst.*, 1947, **84**, 301.

[3] Krebs, E. T., Krebs, E. T., Jr., and Gurchot, C., *M. Rec.*, 1947, **160**, 479.

[4] Council on Pharmacy and Chemistry, *J.A.M.A.*, 1949, **139**, 93.

TABLE I.
Dose and Time Schedule of Chymotrypsin Therapy.

| | | | Chymotrypsin | | | |
|---|---|---|---|---|---|---|
| Case No. | Sex, age | Diagnosis* | Dose, mg | Inj. cumulative, No. | Days treated, No. | Total dose, mg |
| 1 | M-54 | Adenoca. stomach (resected); s.c.met. | 60<br>20 | 7<br>18 | 26 | 640 |
| 2 | M-27 | Anaplastic ca. lung with met. | 20 | 43 | 43 | 860 |
| 3 | M-31 | Adenoca. stomach l.n., s.c. met. | 20 | 60 | 60 | 1200 |
| 4 | M-48 | Epidermoid ca. lung; l.n. met. | 20<br>60 | 27<br>48 | 48 | 1800 |
| 5 | M-72 | Epidermoid ca. alveolus; l.n. met. | 20<br>60 | 49<br>60 | 60 | 1640 |
| 6 | M-54 | Epidermoid ca. esophagus (resected) l.n. met. | 20<br>60 | 43<br>60 | 61 | 1880 |
| 7 | F-65 | Adenoca. breast, l.n., s.c. met. | 60<br>20 | 12<br>39 | 44 | 1260 |
| 8 | M-60 | Adenoca. rectum, liver. l.n. met. | 40<br>20 | 6<br>40 | 40 | 1040 |
| 9 | M-38 | Melanoma, l.n. met. | 20<br>60 | 12<br>53 | 57 | 2700 |
| 10 | M-45 | Melanoma, s.c. met. | 40 | 35 | 38 | 1400 |

* Abbreviations: ca.—Carcinoma. met.—Metastases. l.n.—Lymph node. s.c.—Subcutaneous.

plastic disease received repeated administrations of 20 to 60 mg of crystalline chymotrypsin for 26 to 61 days.† The crystalline material was dissolved in approximately 2 cc of sterile isotonic saline and injected intramuscularly. The first patient initially received 7 doses of 60 mg every other day, but the remaining patients received daily injections with an occasional interruption of one to 3 days.

The patients selected for trial were those with easily palpable or visible lesions or metastases which could be observed and measured objectively for evaluation. Two patients (Cases 1 and 5) had lost considerable weight, but the rest were in fair or good state of nutrition. Two patients (Cases 5 and 7) had received roentgen therapy to the lesions some months previously, and the remainder had had no therapy other than surgery for the primary lesion. Appropriate biopsies were performed before chymotrypsin treatment in all cases, and in 4 patients additional biopsy material was obtained during the course of injections.

In none was there the slightest objective evidence of effect, upon either the neoplastic process or the clinical condition of the patient. No alleviation or decrease of pain, or any subjective improvement were noted. All 10 patients have died after the usual progress of their disease. Necropsies were obtained on 7. No histologic evidence of effect was seen in the biopsy or necropsy sections of the tumors.

All patients receiving chymotrypsin complained of pain and swelling at the site of injection. Sudden severe reactions, consisting of marked diaphoresis, weakness, loss of consciousness, rapid thready pulse, faint heart sounds, and peripheral vascular collapse were encountered in 3 patients, (Cases 8, 9 and 10) within 5 minutes following the 40th, 53rd and 29th doses, respectively, of the enzyme. Prompt use of artificial respiration, epinephrine intramuscularly, oxygen inhalations and intravenous saline probably prevented fatal termination of these anaphylactoid reactions in patients apparently sensitized by previous injections of the protein enzyme. Another patient (Case 6) had a similar but less severe reaction with spontaneous recovery after the 22nd dose.

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† Supplied by Kuster Laboratories, Inc., 571 Seventh Street, San Francisco 3, Calif.

Further trial of chymotrypsin was discontinued, not only because it had no effect whatsoever in 10 patients with advanced malignant neoplasms, but because repeated administrations of the protein enzyme are of definite serious hazard.

*Summary*. Parenteral injections of chymotrypsin had no effect on the course of 10 cases of neoplastic disease in man.

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## 17152. Ineffectiveness of Chymotrypsin Therapy in Malignancy.*

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*From the Department of Biophysics, Medical School, University of California at Los Angeles, and the Birmingham Veterans Administration Hospital, Van Nuys, Calif.*

An elaborate theory has been proposed by Krebs and co-workers[1,2] which assumes that the cancer cell and the trophoblast cell are fundamentally alike, and that both are destroyed selectively by the pancreatic enzyme chymotrypsin. On the basis of this reasoning, but with few supporting facts, chymotrypsin has been suggested as the palliative of choice in neoplastic disease. This report summarizes the negative results of chymotrypsin therapy in experimental and clinical cancer.

*Experimental cancer.* The toxic dose of crystalline chymotrypsin† in mice subjected to repeated daily intraperitoneal injection was established at 40 to 80 mg per kilo of body weight. The dose used in treatment was 2.0 mg per kilo, which is 5 times the recommended dose for humans. Forty mice with subcutaneously transplanted sarcoma 180 and 40 without tumor were employed in the experiment. Half of each group received chymotrypsin intraperitoneally 7 days a week for 14 days, the remainder serving as controls. Weight gains were uniform in all 4 groups of animals. Treatment was begun when tumors were measurable, on the fifth day after transplantation, and tumor size was recorded on alternate days thereafter. The average increase in tumor area at the conclusion of the experiment was 3.8 times in the treated animals and 3.7 times in the controls. No change in chymotrypsin inhibitor concentration of the blood as determined by the method of West and Hilliard[3] resulted from the treatment in either normal or tumor-bearing animals.

*Clinical Cancer.* All patients selected for chymotrypsin treatment had received the maximum benefit from accepted forms of therapy but still had active disease. Their clinical course was followed by an impartial group of observers, Drs. G. M. Leiby, J. A. Weinberg, F. Isaac, and F. W. Wilkins of this hospital. Weekly laboratory determinations included, blood count, sedimentation rate, bleeding and coagulation time, serum protein, alkaline phosphatase, cephalin flocculation, thymol turbidity, NPN, and serum chymotrypsin and rennin inhibitors.[3] When feasible, patients were followed roentgenographically as well.

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* Aided by a grant from the California Institute for Cancer Research. Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

[1] Gurchot, C., Krebs, E. T., Jr., and Krebs, E. T., *Surg., Gynec. and Obst.*, 1947, **84**, 301.

[2] Krebs, E. T., Krebs, E. T., Jr., and Gurchot, C., *M. Rec.*, 1947, **160**, 479.

† Crystalline Chymotrypsin was supplied by Spicer-Gerhart Co., Pasadena, Calif., and Worthington Biochemical Laboratory, Freehold, N. J.

[3] West, P. M., and Hilliard, Jessamine, in press.

The diagnosis in each instance had been proved by biopsy. There were 15 cases in the series: bronchogenic carcinoma, 3; carcinoma of stomach, 2; embryonal carcinoma of testicle, 2; and one each of recto-sigmoid and uterine carcinoma, synovioma, lymphosarcoma, Hodgkin's disease, acute and subacute lymphatic leukemias, and chronic myelogenous leukemia.

Crystalline chymotrypsin in saline was given in daily intramuscular doses ranging from 4 to 60 mg for periods of 21 to 135 days, with an average of 64 days. The usual dose was the recommended one of 30 mg. Three patients could not tolerate more than 4 to 6 mg without intense pain in the most involved organ (liver, stomach and spleen) accompanied by chills and fever 12 to 24 hours after injection. Six cases developed pronounced allergy to the drug, forcing reduction in dose or discontinuation of treatment. Two had generalized urticarial reactions, 2 exhibited anaphylactic shock, and 2 had extreme local erythema and edema at the site of injection. Two patients died in congestive failure about a week after onset of precordial pain, ankle edema and dyspnea. Electrocardiograms were normal before death and autopsy failed to reveal significant cardiac pathology. Whether or not these deaths were related to treatment is debatable.

The sedimentation rate was doubled during chymotrypsin therapy. There were no other consistent laboratory findings. The serum level of chymotrypsin inhibitor was not altered detectably, which is understandable in view of the fact that there is sufficient inhibitor in the circulation alone in many such patients to inactivate 25 to 50 g of pure chymotrypsin.

In no case was the clinical course of the patient influenced by chymotrypsin therapy. Nine patients have died and a study of autopsy material has not revealed any unusual degenerative changes in the neoplasm.

*Summary.* Treatment with crystalline chymotrypsin did not influence the course of neoplastic disease in experimental animals or in fifteen selected cancer patients.

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## 17153. Action of Chloromycetin on Salmonella.

Erich Seligmann and Michael Wassermann. (Introduced by William Antopol.)
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As yet there exists no effective remedy to prevent or cure Salmonella infections in man and animals. Any new antibiotic with selective activity against gram negative organisms, therefore, is worth a trial against Salmonella infections. Chloromycetin, the antibiotic produced by Streptomyces Venezuelae[1] possesses these qualities in addition to its marked effect against Rickettsia. Clinical experiments indicate some success in the treatment of human typhoid fever (a Salmonellosis.)[2]

*In vitro* tests with a number of gram negative bacilli were performed by Smith *et al.*,[3] and revealed remarkable sensitivity, usually in a range below 1 $\mu$g of the drug. These results were obtained by a special turbidity assay method developed by Joslyn and Galbraith.[4] A strain of *Sh. sonnei* No. 04628 was used as the test strain, and inhibited by .2 μg in this set-up. In our experiments* the same test strain was employed in a simplified serial dilution method. Sensitivity was measured by the smallest amount of chloromycetin that completely suppressed growth after 24 hours. With this method the test strain was regularly sensitive to 1 μg.

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[1] Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

[2] Woodward, T. E., Smadel, J. E., Ley, H. L., Jr., Green, R., and Mankikar, D. S., *Ann. Int. Med.*, 1948, **29**, 131.

[3] Smith, R. M., Joslyn, D. A., Gruhzit, O. M., McLean, I. W., Jr., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, **55**, 425.

Sixty-eight gram negative strains were examined; they included *B. coli* and other coliform organisms, *aerobacter aerogenes, B. proteus, Pseudomonas aeruginosa, Shigella* types, 2 strains of *S. typhosa* and 21 other Salmonella types. Most strains of *Pseudomonas aeruginosa* and some coliform organisms were remarkably resistant to chloromycetin and required concentrations from 20-100 μg per ml for inhibition. Of the 23 Salmonella types, 19, including *S. typhosa,* were sensitive to 2 μg, four to 4 μg, none higher. No relation existed between streptomycin resistance and chloromycetin resistance; no absolute resistance to chloromycetin was encountered in any strain.

*For animal experiments* (oral and parenteral treatment) solutions of chloromycetin in 20% propylene glycol were used; emulsions in acacia, as recommended, were not practicable because of the small amount of the material to be ingested.

Crystallized chloromycetin is not readily soluble in water; suitable solutions however, can be produced by use of 20% propylene glycol. Final concentrations of 10 mg per ml were obtained by heating for 10-20 minutes in a water bath at 56°. At room temperature needle-shaped crystals precipitated. They disappeared on repeated immersion into the water bath at 56°. The antibiotic activity of these solutions was not impaired by repeated exposure to 56°. Mice, 20 g of weight were fed with chloromycetin solution by introduction through a smoothly blunt 18 gauge needle attached to a tuberculin-syringe. For 4 consecutive days 2000 μg (contained in .2 ml) were given twice daily, at 9 A.M. and 4 P.M. The total dose amounted to 16 mg or 800 mg per kg mouse. No toxic effects were observed.

The intestinal flora was checked by stool cultures taken before the start of the experiment and after the 5th and 7th feeding. No growth difference was found; number and size of colonies (mostly *B. coli, B. proteus,* enterococci), remained almost unchanged during the course of the tests. In this respect the action of chloromycetin is different from that of streptomycin. Feeding of the latter drug results in a marked decrease of fecal organisms, even bringing about complete inhibition of growth.[5,6] Streptomycin remains practically unabsorbed in the intestinal tract, and is not destroyed in the intestines. Since chloromycetin is readily absorbed by the oral route,[3] no active material remains in the intestines for any length of time. The fecal flora, therefore, is not affected.

*Salmonella infections.* Virulent organisms used for infection of mice by the oral or parenteral route, as a rule, give rise to generalized septicemia with fatal outcome. The bacilli transgress the intestinal wall from both sides, from the intestinal into the general circulation as well as from blood and lymph system into the intestinal lumen. The failure, therefore, of chloromycetin given by mouth to influence the bacteria inside the intestinal tract does not preclude its possible activity against those Salmonella organisms that have entered the body system from there.

In the first experiment 40 mice of about 20 g body weight received orally .1 cc of an 18 hour broth culture of *S. typhi murium* 1908 known for its constant virulence. Twenty animals served as controls, 10 mice underwent oral treatment: 2000 μg twice a day for 4 days, once a day for 4 more days; total ingested chloromycetin was 24 mg or 1200 mg per kg. A second batch of 10 mice was injected subcutaneously with 2500 μg twice a day for 6 days and once a day for 2 more

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[4] Joslyn, D. A., and Galbraith, M., *J. Bact.*, 1947, **54**, 26.

* Crystallized chloromycetin was supplied through the courtesy of Parke Davis and Co.

[5] Seligmann, E., and Wassermann, M., *J. Bact.*, 1946, **53**, 127.

[6] Seligmann, E., Barash, L., and Cohlan, S. Q., *J. Ped.*, 1947, **20**, 182.

TABLE I.
Oral Infection of Mice with 0.1 cc of Broth Culture of *S. typhi murium* 1908 and Treatment with Chloromycetin.

| Treatment | No. of mice | Surviving after, days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 14 | 21 |
| None (controls) | 20 | 20 | 16 | 12 | 9 | 6 | 3 | 1 | 0 | | | |
| Oral | 20 | 10 | 10 | 10 | 10 | 8* | 7 | 7 | 5 | 5 | 2 | 1 |
| Parenteral | 10 | 10 | 10 | 10 | 10 | 9* | 9 | 9 | 8 | 7 | 6 | 1 |

* Last day of treatment.

TABLE II.
Parenteral Infection of Mice with 0.1 cc of Broth Culture of *S. typhi murium* 1908 (1:60) and Treatment with Chloromycetin.

| Treatment | No. of mice | Surviving after, days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 14 | 21 |
| None (controls) | 20 | 20 | 7 | 5 | 4 | 0 | | | | | | |
| Oral | 10 | 10 | 10 | 10 | 10 | 8* | 7 | 7 | 5 | 5 | 2 | 1 |
| Parenteral | 20 | 20 | 20 | 18 | 15* | 11 | 10 | 9 | 6 | 5 | 3 | 3 |

* Last day of treatment.

days. Total injected chloromycetin was 35 mg or 1750 mg per kg. Table I gives the results.

Treatment began immediately after infection and terminated on the eighth day. On the 11th day all controls were dead, while 5 and 8, respectively, of the treated animals were still alive. At the end of the experiment, however, (21st day) only one of each series of treated animals survived. Thus, oral infection with *S. typhi murium* could not be checked by oral or subcutaneous treatment with large doses of chloromycetin. In Table II similar experiments were performed after parenteral infection of the mice (.1 cc of an 18 hour broth culture of *S. typhi murium* 1908, diluted 1:60). The parenteral treatment with chloromycetin was performed for 7 days with a total of 25 mg or 1250 mg per kg; the oral treatment (8 days) with a total of 24 mg or 1200 mg per kg.

The results are almost identical with those of the first test. When all untreated animals had succumbed (7th day), a considerable number of treated mice were still alive. On withdrawal of the drug, however, and in the course of the next 2 weeks, most of the treated animals died. Only a few survived at the end of the experiment (21st day). *S. typhi murium* was recovered from the controls and the treated dead mice, often in pure culture. It was found in heart blood and intestines in both series. The chloromycetin sensitivity of the recovered strains had not changed. The original culture, as well as the strains isolated from the animals, still showed sensitivity to 2 μg.

*Summary.* Chloromycetin inhibits the growth of Salmonella organisms *in vitro* remarkably, but is not able even in large amounts to control Salmonella infections in mice. Neither the oral nor the subcutaneous form of treatment prevented the fatal outcome of the disease in most animals, although it prolonged the survival time. Its action in the animals was bacteriostatic and not bactericidal. Due to its absorption conditions, chloromycetin exerts no influence on the intestinal flora, when given orally.

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# 17154. Small-nerve Fibers in Mammalian Ventral Roots.*

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The function of the frogs small-nerve motor system has been studied in detail during the last few years.[1-4] Slowly conducting high threshold nerve fibers of around 5 $\mu$ in diameter emerge through the ventral roots and innervate numerous muscles in the body. On stimulation they cause local graded contractions around the neuromuscular junction, depending on the frequency of stimulation. The frogs small-nerve system also shows a distinct reflex pattern.

In the cat about 25 per cent of the nerve fibers in the ventral roots of the lumbosacral region are medullated fibers of small diameter with a distribution peak of 4-6 $\mu$.[5] Their physiological properties have been studied by several investigators.[4,6,7] In particular Leksell[8] concluded that the effect of the small-nerve fibers on the contractile mechanism was small or absent and that the fibers influenced the afferent discharge from muscle proprioceptors.

A systematic investigation was made of the function of ventral root nerve fibers to the soleus and tenuissimus. By subdividing ventral roots into fine filaments individual nerve fibers innervating different muscles were obtained. The nerve fibers were then stimulated singly or together at varying frequencies and their effect was determined on the contractile and sensory mechanism of the muscles. Nerve fibers conducting at speeds faster than 50 m/sec caused the well known motor unit response, *i.e.* twitches and propagated muscle impulses. Nerve fibers conducting between 16-50 m/sec. caused no detectable muscle shortening and no propagated muscle impulses. Calculations of diameters from conduction velocities in the small-nerve group showed a distribution in agreement with histological data (3-8 $\mu$, cf[5]).

The sensory discharges of muscles could be detected by recording from the surface of the tenuissimus, from nerve branches emerging from muscles, or preferably by isolating filaments of dorsal roots, containing a single nerve fiber from the muscle under study. The latter method gave simple and clear-cut indication of the sensory discharge regulation in muscle spindles, since a single efferent fiber to a muscle spindle could be stimulated and its effect recorded in a single sensory fiber. Different discharge patterns discovered by Matthews[9] were easily detected and his findings of $A_1$, $A_2$ and B discharge types were readily confirmed in numerous observations.

Stimulation of the small ventral root fibers, when effective, increased the afferent discharge from muscle spindles, dependent upon the tension of the muscle and the duration and frequency of stimulation. The increase could be elicited from spindle receptors which showed a steady rate of discharge or from receptors which showed no resting discharge at a given initial tension. The principal feature of the small-nerve mechanism is illustrated in Fig. 1, which shows that the sensory discharge can be finely regulated by adjustment of muscle tension and by motor excitation of the spindle intrafusal fibers. In A, B, and C it is shown that the discharge is not directly proportional to the

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* Supported by a grant from the National Foundation for Infantile Paralysis, Inc., and by the Rockefeller Foundation.

† Senior Fellow of the National Research Council.

[1] Kuffler, S. W., and Gerard, R. W., *J. Neurophysiol.*, 1947, **10**, 383.

[2] Kuffler, S. W., Laporte, Y., and Ransmeier, R. E., *J. Neurophysiol.*, 1947, **10**, 395.

[3] Tasaki, I., and Mizutani, K., *Jap. J. med. Sci.*, 1944, **10**, 237.

[4] Tasaki, I., and Tsukagoshi, M., *Jap. J. med. Sci.*, 1944, **10**, 245.

[5] Eccles, J. C., and Sherrington, C. S., *Proc. Roy. Soc.*, 1930, **106B**, 326.

[6] Häggquist, G., *Acta Med. Scand.*, 1940, **104**, 8.

[7] O'Leary, J., Heinbecker, P., and Bishop, G. H., *Am. J. Physiol.*, 1934, **110**, 636.

[8] Leksell, L., *Acta physiol. Scand.*, 1945, **10**, suppl. 31, 84 pp.

[9] Matthews, B. H. C., *J. Physiol.*, 1933, **78**, 1.

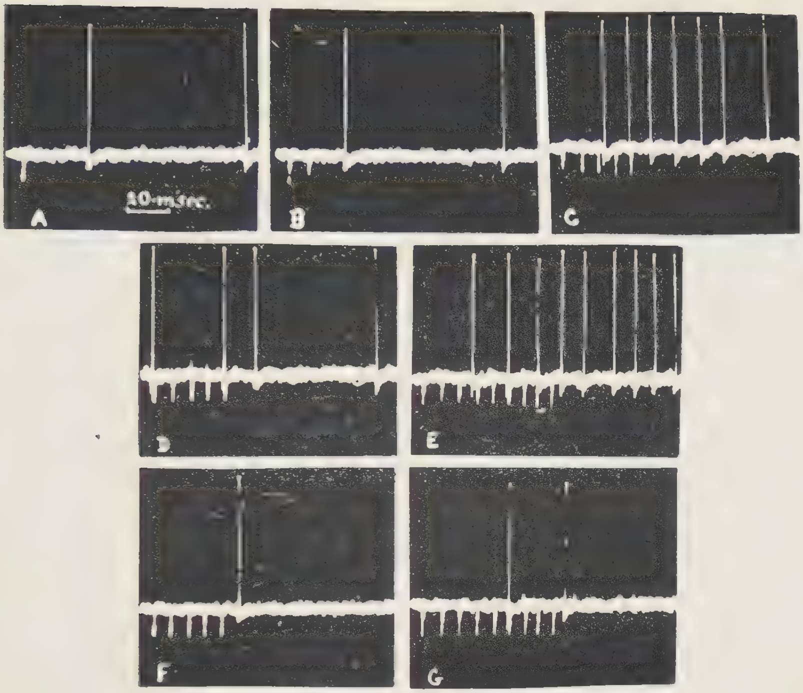

FIG. 1.

Small-nerve fiber stimulation in ventral root, recording from dorsal rootlet. Single fiber afferent discharges from soleus at varying muscle tensions. Stimulus intervals are 9 msec.

A, B, and C—Muscle tension 40 g. In A and B 2 and 3 stimuli set up 2 sensory spindle discharges. C. 6 stimuli cause a train of responses.

D and E—Tension 20 g. D. 6 stimuli as in C now set up fewer discharges. E. 10 stimuli restore longer train of discharges.

F and G—Zero tension. 6 and 10 stimuli are practically ineffective. Occasional resting discharges occurred at 40 and 20 g muscle tension.

number of stimuli but shows a mechanism of facilitation. Lowering the muscle tension renders the spindle less sensitive to small-nerve excitation. but at intermediate tensions an increase in number of stimuli can compensate for such an effect (C + E). However, at zero muscle tension even prolonged stimuli remain practically ineffective. The spindle of Fig. 1 gave an $A_1$ type response as described by Matthews. Its discharge showed a high threshold to stretch.

The facilitation mechanism of spindle discharge and other data suggest that the small-nerve fibers in the cat cause local contractions in intrafusal muscle fibers. This would be analogous to the graded contractile responses in frog striated muscles, innervated by the small-nerve motor system.

Besides the small-nerve innervation described here, many spindle intrafusal fibers are supplied by motor nerves of larger diameter as originally found by Matthews (cf also[10]). The latter cause a discharge pattern which is in contrast to the one illustrated in Fig. 1 in that a single nerve impulse sets up a burst of sensory discharges.

*Summary.* The lumbosacral ventral root outflow of the cat contains 20-25 p.c. small diameter nerve fibers. These cause no detectable shortening of muscles and no propagated muscle impulses. Stimulation of such fibers sets up discharges from muscle spindle receptors. The discharge is dependent upon the number and frequency of stimuli and on adjustment of muscle stretch.

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[10] Barker, D., *Quart. J. Microscop. Sci.*, 1948, **89**, 143.

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# 17155. Staphylocoagulation in Plasmas of Various Animal Species.*

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Smith and Hale[1] showed that clotting of decalcified plasma by staphylococci takes place in two stages, namely, (1) reaction of the bacterial product (prostaphylocoagulase[2]) with a cofactor or activator-substance in plasma to form the active clotting agent (staphylocoagulase), which (2) then converts fibrinogen into a fibrin which, according to our own observations under the dark-field microscope, appears indistinguishable from ordinary fibrin, despite the fact, which we have indubitably confirmed, that the staphylcoagulation phenomenon can occur in the complete absence of the thrombin mechanism.[2] We have previously reported[3] on the occurrence of cofactor (Tager's[4,5] "coagulase reacting factor") in various blood and tissue products, finding a human serum albumin (see reagents) to be the most satisfactory source material.

In earlier literature on the great variability of the staphylocoagulation phenomenon in plasmas of differing animal species, some suggestions have been made as to lack of cofactor (activator), thermolability of activator substance or of staphylocoagulase, and possible combinations of these and other factors. For instance, it has been stated[1] that fowl and mouse plasmas completely lack activator substance, whereas guinea-pig plasma clots at 20°C but not at 37° owing to low concentration of activator, in consequence of which staphylocoagulase production proceeds at too slow a rate to keep pace with thermal deterioration at the higher temperature (cf. infra). Kaplan and Spink[6] give evidence for a further factor, namely, an inhibitor, demonstrable in the euglobulin fraction of human and guinea-pig plasmas. It is precipitable between 25 and 40% saturation with ammonium sulfate. Tager and Hales[5] also suggest that the coagulase reacting factor may be bound or masked in the plasma of certain species.

The present study is an extension of earlier reports[2,3] and describes experiments in which the plasmas of a number of animal species have been tested with some modified methods and fractioned with classical ammonium sulfate procedures in an attempt to explain more fully the reasons for reduction or absence of staphylocoagulation in the case of certain species.

*Reagents.* 1. The borate buffer, pH = 7.7, used as solvent and diluent throughout, was citrated to 0.4% $Na_3C_6H_5O_7$, 5½ $H_2O$; 2. a 1% staphylococcal product, containing *both* prostaphylocoagulase and staphylokinase, was prepared as described previously;[3] 3. Fibrinogen was Armour's bovine plasma Fraction-I; 4. Thrombin (10 units/ml) was diluted commercial (Parke, Davis) "Thrombin Topical"; 5. The plasmas of the various species were centrifuged in citrate (0.4%) and, for some tests, fractioned with ammonium sulfate into the classical crude "globulins" and "albumin" as follows: I. 50% saturation with $(NH_4)_2SO_4$ precipitated the globulins which, on dialysis until sulfate-free yielded (a) "euglobulin" precipitate: dissolved in citrated borate buffer to original plasma volume, (b) "pseudoglobulin" supernatant; dialyzed and concentrated *in vacuo* to original plasma volume;

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[2] Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Fed. Proc.*, 1948, **7**, 41.

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TABLE I.
Staphylocoagulability of Various Plasmas, at 37°C and 20°C. Clotting-times with Prostaphylocoagulase; A. with, B. without, Added Cofactor.

| Plasma | A. Prostaph. + cofactor | | B. Prostaph. + buffer | |
|---|---|---|---|---|
| | 37°C | 20°C | 37°C | 20°C |
| Rabbit | 19 min. | 22 min. | 18 min. | 17 min. |
| Human | 36 min. | 54 min. | 36 min. | 54 min. |
| Chicken | No clot* | No clot* | No clot* | No clot* |
| Rat | ” ” | ” ” | ” ” | ” ” |
| Guinea pig | 240 min.† | 324 min. | No clot‡ | 323 min. |
| Dog | 73 min. | 29 min. | 180 min.† | 28 min. |
| Horse | 100 min. | 235 min. | 227 min. | 228 min. |
| Bovine | 150 min. | 660 min. | 660 min. | 660 min. |

* Thrombin added after 24 hr gave solid clot; no fibrinogenolysis.
† Clot not quite solid; fibrinolysis subsequently.
‡ Thrombin added after 24 hr gave no clot; fibrinogenolysis.

II. albumin supernatant from the original globulin precipitation: similarly dialyzed and concentrated to original plasma volume; 6. cofactor (activator) of prostaphylocoagulase was *human* serum albumin prepared as above.

*Factors affecting staphylocoagulation in plasmas of various species.* Tests were run at 20°and 37°C, timing the coagulation of 0.5 ml plasma with A. 0.25 ml prostaphylocoagulase + 0.25 ml cofactor; B. 0.25 ml prostaphylocoagulase + 0.25 ml buffer. When no clotting was evident after 24 hours, 0.25 ml thrombin was then added to determine whether fibrinogenolysis had occurred incidental to the staphylokinase-protease phenomenon. In order to determine inhibitory effects directed against *active* staphylocoagulase, prostaphylocoagulase and cofactor were incubated at 37° for half an hour before adding the selected plasma. Thus, species differences in staphylocoagulation times could be evaluated, at least semiquantitatively, with respect to (1) low cofactor content; (2) presence of inhibitors (s); 3. interference by protease, or (4) several possible combinations of these factors. The initial data are summarized in Table I, and their analysis is supplemented by the special tests of Tables II and III.

These experiments indicate: 1. Staphylocoagulation times, under carefully controlled test conditions, vary according to species of plasma; 2. Several plasma factors contribute to these species variations, *e.g.* (A) Rabbit, dog, human, and to a lesser extent horse, guinea pig, and bovine, give staphylocoagulation with the bacterial product alone (the citrate precluding any possible thrombic mechanism) and must, therefore, contain cofactor: (B) additional cofactor reduces the coagulation times of horse and bovine plasma, but not to the level of the best clotting times seen with rabbit plasma. Proteolysis is not evident in these species. Hence it is concluded that they are relatively lower in cofactor than the rabbit and also contain some inhibitor: (C) Human plasma gives the same staphylocoagulation times, with or without cofactor, and longer than those for rabbit plasma. In the absence (in these particular experiments) of proteolytic effects, the explanation must be in terms of inhibitor(s): (D) That at least part of the inhibitory effects is against active staphylocoagulase is proved by the method stated. Such inhibitor is very marked in chicken and rat plasmas which do not clot under any of the test conditions, including the addition of preincubated prostaphylocoagulase + cofactor. Since clots occur on the addi-

TABLE II.
Inhibitory Effects of Various Plasma Dilutions; 0.25 ml Added to Mixture*: 1 ml Fibrinogen, 0.5 ml Cofactor, 0.25 ml Prostaphylocoagulase. Clotting-times (min.) at 37°C.

| Species | Plasma dilutions | | | | |
|---|---|---|---|---|---|
| | 1:1 | 1:8 | 1:32 | 1:128 | 1:256 |
| Chicken | 2160 | 119 | 44 | 44 | 42.5 |
| Horse | 318 | 62 | 62 | 40 | 40 |
| Guinea pig | 80 | 80 | 64.5 | 64.5 | 41 |

* Control (buffer instead of plasma): 40 min. staphylocoagulation time.

TABLE III.
Tests for Inhibitor and Cofactor.

| Species plasma | Inhibitor | | | Cofactor | | |
|---|---|---|---|---|---|---|
| | Euglob. | Pseudoglob. | Alb. | Euglob. | Pseudoglob. | Alb. |
| Chicken | + | 0 | + | 0 | ± | + |
| Horse | ± | + | 0 | + | + | + |
| Guinea pig | ?<br>(lysis) | 0 | 0 | ?<br>(lysis) | 0 | + |

tion of thrombin after 24 hours, fibrinogenolysis is not significant in these cases: (E) With guinea-pig plasma, our findings are staphylocoagulation at 20°C in a time which is the same whether or not cofactor is added. The last is not a variable, therefore. Nevertheless, at 37°C, clots obtained in the presence of cofactor are not quite solid and undergo fibrinolysis subsequently. Without cofactor, no clot is obtained at 37°C and the 24 hr. test with thrombin is negative, showing that fibrinogenolysis has occurred. Our experiments are a partial confirmation of those of Smith and Hale[1] but complete the explanation in terms of interfering effects of proteolysis induced by the accompanying staphylokinase[7,8] factor in the staphylococcal product, with no need to postulate any instability of the staphylocoagulase. The fact that in guinea-pig plasma staphylocoagulation is faster at 37° than at 20° confirms Kaplan and Spink,[6] but these workers' data are probably more significant as to temperature coefficient since their staphylococcal product would appear to have been deficient in staphylokinase: (F) in dog plasma, proteolysis, at 37°, is also encountered but the staphylokinase interference is less, so that some staphylocoagulation still occurs. That the clotting is slower at the higher temperature, in this case, does indicate greater interference by the proteolysis as the temperature is raised from 20° to 37°C. Moreover, it is slower, at 37°C, when cofactor is not added. Owing to interference by fibrinogenolysis it can be suggested only tentatively that dog plasma has more cofactor than guinea-pig's and less than rabbit's.

*Inhibitory effects of various plasmas on staphylocoagulation.* In order to investigate further the inhibitory phenomena, the special tests summarized in Table II were performed on plasmas selected from chicken, horse, guinea-pig, *i.e.* representative, respectively, of 1. antistaphylocoagulase, 2. inhibitor accompanying lesser amount of cofactor, 3. lesser cofactor with accompanying proteolysis. Tests were performed at 37° C on mixtures of fibrinogen (bovine, 1 ml), cofactor (human albumin, 0.5 ml), and prostaphylocoagulase (0.25 ml). To this 0.25 ml of various plasma dilutions was added. As compared with a 40 minutes staphylocoagulation time in the control (buffer instead of plasma), definite inhibitory effects were apparent in all cases and lessened on dilution. The inhibitor is highest in undiluted chicken plasma. Proteolytic effects did not significantly interfere when guinea-pig plasma was tested under these conditions.

*Cofactor and inhibitor of staphylocoagulation in various plasma fractions* : The euglobulin, pseudoglobulin and albumin fractions of the plasmas of the above three species were tested: 1. in a repetition of the foregoing demonstration of inhibitor, using only the full strength plasma fraction, however; 2. in a similar test system but omitting the human albumin (cofactor) in order to detect the presence of cofactor in the various plasma fractions. The data are given, qualitatively, in Table III.

We have noted that no fraction yields an inhibitor concentration comparable to that of the original plasma and, in fact, the total inhibition by all fractions does not equal that of the whole original plasma. Owing to proteolysis, it is not possible to be certain of the inhibitory effect of guinea-pig euglobulin. Its

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[7] Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 246.

[8] Lack, C. H., *Nature*, 1948, **61**, 559.

pseudogloulin and albumin are devoid of inhibitor, as are the pseudoglobulin of chicken and albumin of horse plasma. Confirming our earlier work,[3] the albumins are found to contain most of the cofactor. Positive results are obtained, however, with euglobulin of horse and pseudoglobulin of horse and chicken (weak). Negative results are found only with guinea-pig pseudoglobulin and chicken euglobulin. Guinea-pig euglobulin could not be tested with certainty owing to fibrinogenolysis. Thrombin interferes with tests on euglobulin in similar experiments with human and some other species of plasma.

*Summary.* Marked species variations in the staphylocoagulation phenomenon are encountered and can be explained in terms of several factors. Comparing with rabbit plasma as an empirical standard (shortest staphylocoagulation times encountered), the ability to shorten staphylocoagulation time by addition of cofactor (human serum albumin) is evidence for lesser amounts of cofactor in such plasmas as horse and bovine. Cofactor cannot be recovered completely or uniformly in plasma fractions obtained with the classical $(NH_4)_2SO_4$ salting procedures. Nevertheless, the "albumin" is consistently rich in cofactor (for activation of prostaphylocoagulase) and devoid of thrombin (which interferes in tests on some euglobulins).

Excess of inhibitor(s), in part at least directed against active staphylocoagulase as shown by pre-incubation of prostaphylocoagulase with cofactor before adding to plasma (or plasma dilution + fibrinogen) is a feature of some plasmas, notably rat and chicken. Owing to this, the demonstration of cofactor requires plasma fractionation, in these instances.

Fibrinogenolytic and possibly other proteolytic effects may interfere with tests, especially at higher (37°C) temperatures, and are exemplified in guinea-pig and dog plasmas.

The quantitative evaluation of staphylocoagulation times must, therefore, take cognizance of variability not only in the two bacterial factors but, even more significantly, in the various plasma factors on which they operate in the systems devised for testing the staphylocoagulation and proteolytic[9] phenomena. There are marked species differences in these plasma factors.

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[9] Gerheim, E. B., and Ferguson, J. H., Proc. Soc. Exp. Biol. and Med., following paper.

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## 17156. Species Reactivity to Staphylokinase.*

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The mechanism of staphylococcal fibrinolysis has been shown recently[1,2] to be analogous to the mechanism of streptococcal fibrinolysis,[3] namely, a kinase type of activation by the bacterial product, of plasma or serum proenzyme (protease precursor) to yield the active lytic agent. In an earlier study,[2] of which the present data are an extension, the distinction between the bacterial factor taking part in proteolysis (*staphylokinase*) and that involved in coagulation (*pro-Staphylocoagulase*)[4] was made by means of

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† Predoctorate Research Fellow, U. S. Public Health Service. Present address: Wayne University College of Medicine, Department of Physiology and Pharmacology, Detroit, Mich.

[1] Lack, C. H., *Nature*, 1948, **161**, 559.

[2] Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 246.

[3] Milstone, H., *J. Immunol.*, 1941, **42**, 109.

[4] Gerheim, E. B., and Ferguson, J. H., Proc. Soc. Exp. Biol. and Med., previous paper.

TABLE I.
Staphylococcal Fibrinolysis and Inhibition by Crystalline Soybean Trypsin-inhibitor, in Plasma Clots of Various Species. 37°C; pH = 7.7 (borate buffer).

| Species | I. Staphylokinase | II. Staph. + inhibitor |
|---|---|---|
| Dog | 25 min. | 60 min. |
| Human | 50 min. | 165 min. |
| Guinea pig | 60 min. | 225 min. |
| Rabbit | 270 min. | No lysis (48 hr) |
| Ox | No lysis (72 hr) | No lysis (72 hr) |
| Horse | ,, | ,, |
| Sheep | ,, | ,, |
| Rat | ,, | ,, |
| Chicken | ,, | ,, |

heat lability experiments. The current study concerns (1) species differences in plasma factors which react with staphylokinase, (2) differences between staphylokinase and streptokinase (a) in these species plasma reactivities and (b) in the rates with which they induce their respective activation of protease precursor.

*Reagents.* 1. Plasmas were collected from the various animal species by centrifugation of blood received into one-tenth volume of 4% sodium citrate (hydrated); 2. Thrombin was the commercial (Parke, Davis) "Thrombin Topical" diluted to 10-15 units/ml; 3. Fibrinogen was Armour's bovine plasma Fraction-I, which was proenzyme-free[5] inasmuch as no active protease could be detected after treatment with (1) chloroform, (2) bacterial kinases, or (3) "fibrinokinase" of Astrup and Permin;[6] 4. The staphylococcal product, containing both *staphylokinase* and *prostaphylocoagulase* (of no consequence in the presence of added thrombin) was prepared in 1% solution as previously described;[7] 5. Crystalline soybean antiprotease (trypsin-inhibitor), courtesy of Dr. M. Kunitz,[8] was used in 1:10,000 solution; 6. Borate buffer,[5] pH = 7.7, was used as solvent and diluent throughout.

*Staphylokinase- and streptokinase-induced fibrinolysis in plasma clots of various species.* Confirming earlier workers,[9] we find *streptokinase* to lyse human plasma (fibrin) clots, but unable to induce fibrinolysis in plasma of other animal species.

*Staphylokinase* is tested in the following systems: I. 0.5 ml citrated plasma + 0.25 ml staphylokinase + 0.25 ml buffer + 0.25 ml thrombin; II. similar mixtures but containing 0.25 ml 1:10,000 crystalline soybean antiprotease instead of buffer. The data given in Table I are representative of a number of experiments.

Staphylokinase does not show the strict species specificity of streptokinase, but does fail to lyse plasma clots (Table I) of ox, horse, sheep, rat, and chicken. Fibrinolysis is obtained, however, with dog, human, guinea-pig, rabbit. Moreover, the respective lysis times are usually in that order both in I. and in II. In the latter series, a weak protease inhibitor is chosen to bring out its relative inhibitory effects and these show a definite correspondence with the initial enzyme potency. Thus, in the case of the weak rabbit protease the inhibitor inhibits fibrinolysis for at least 48 hours, whereas its effect is relatively minor, *i.e.* 60 min. *vs.* 25 min., on the lysis time in dog plasma tests.

In repeated tests on a single species, *e.g.* rabbit plasma, considerable variations in lysis time are encountered, despite the use of the same staphylokinase preparation and carefully standardized test conditions. It may be recalled that Lack[10] consistently failed to activate rabbit plasma with his staphylokinase preparations and hence suggested possible plasma inhibitors.

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[9] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

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The working out of quantitative methods to estimate and distinguish between plasma differences as to enzyme precursor, on the one hand, and the possible inhibitors, on the other, is beyond the scope of the present communication.

It is, nevertheless, possible to conclude that, in addition to individual plasma variations, there are wider and more significant species variations, clearly illustrated by the data of Table I, which, whatever the ultimate cause, do represent definite species differences in the plasma reactivity of staphylokinase.

Staphylokinase fibrinolysis in human plasma Fraction-I was noted in a previous paper.[2] Clearly, staphylokinase and streptokinase can only be compared on human plasmas or products containing the species reactive proenzyme and equivalent in terms of such still ill-defined factors as the possible antikinase and antiprotease inhibitors.

*Rates of activation of proenzyme by staphylokinase and streptokinase.* Numerous experiments in our laboratory confirm the conclusion of previous workers[11,12] that streptokinase activates human plasma proenzyme "almost instantaneously" (Kaplan, 1945). In such case, attention is directed away from the fact that the events following the addition of bacterial kinase to plasma clot systems occur in two phases, namely, *first* the activation of proenzyme to lytic agent, and only *secondly* lysis of the substrate (fibrin). Early in our studies of staphylokinase the question was raised whether this activator might not differ from streptokinase in the rapidity of the first phase reaction. The following test system was, therefore, devised to permit independent following of the progress of the activation of proenzyme. Serum (4 ml) was used as a fibrinogen-free source of proenzyme and incubated at 37°C with the staphylococcal product (1 ml). After suitable incubation periods, 0.5 ml of the mixture was added to 1 ml proenzyme-free bovine fibrinogen and immediately clotted with 0.5 ml thrombin. Such experiments were performed on human, rabbit, guinea-pig, and dog sera. Typical results from sera of the last two species are recorded in Fig. 1. It may be seen that the shortest lysis times (optimal protease activity) required an incubation averaging 30-60 minutes. In the experiment with dog serum, the lysis test after 15 seconds incubation was 33 minutes, but reached an optimal of 4 minutes lysis time after incubation for half an hour. Thereafter, the lysis times gradually lengthened, reaching 36 minutes after 4 hours of incubation, obviously denoting instability or inactivation of the enzyme at the 37° temperature. The guinea-pig data are less striking but qualitatively similar.

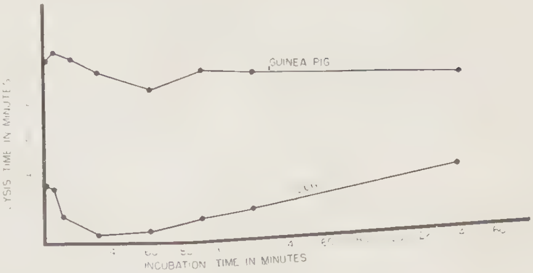

Fig. 1.
Activation of serum proenzyme by staphylokinase.

*Summary.* Fibrinolytic tests are described which clearly demonstrate differences in the species reactivity of plasmas to the staphylokinase and streptokinase activation of the protease system. Whereas streptokinase acts only on human materials, staphylokinase works on plasma (or serum) of dog, guinea-pig, and rabbit also, but gives negative results with bovine and other species tested. Staphylokinase differs from streptokinase also in requiring an incubation period of many minutes before reaching optimal activation of the serum proenzyme.

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## 17157. Effect of Testis Extract on Growth of a Transplanted Lymphosarcoma in AKm Mice.*

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(Introduced by C. P. Rhoads).

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Mammalian testis extract, as well as many bacteria, contains a factor which enhances the *in vivo* spread of dyes, infective organisms and toxins. This substance is accepted to be identical with the enzyme hyaluronidase.[1] Since the first description of the spreading factor by Duran-Reynals,[2] a number of workers have considered the possible relationship between this factor and the local and distant spread of malignant growths. Such investigations have sought to determine, 1) the actual content of spreading factor in tumors, as demonstrated by *in vivo* and *in vitro* methods, and 2) the behaviour of tumor transplants under the influence of the factor. Duran-Reynals and Stewart[3] demonstrated the spreading factor in some human carcinomas, but not in sarcomas. McCutcheon and Coman[4] also offered evidence of the spreading factor in human carcinomas. In a series of animal tumors Boyland and McClean[5] found a great variation in spreading activity, with the highest values in mouse and fowl sarcomas. The concentration of spreading factor in tumors never approached that of normal testis tissue. Hakanson and Glick[6] reported an elevation in the anti-hyaluronidase titer in sera from patients with malignant tumors. This increase was more marked in cases of metastatic than of non-metastatic tumors. Coman, McCutcheon and Zeidman[7] gave several injections of hyaluronidase in or around transplanted mouse Sarcoma 241, and Shope Papilloma, but observed no effect on the growth of the tumors.

When the malignant transplants were combined with testis extract prior to inoculation, Duran-Reynals[8] found that the growth of the Brown-Pearce carcinoma was retarded or prevented. In his discussion he favors the explanation that this phenomenon is due to the increased cellular and tissue permeability brought about by the spreading factor. He states, however, that this unitarian interpretation of the effects of testis extract is not positively established. Using a similar technic, Prime and Haagensen[9] could not demonstrate any effect on a transplanted rat sarcoma, a rat carcinoma or a guniea pig sarcoma. Hoffman, Parker and Walker[10] were able to show that rabbit testis extract enhanced the spread of the Chicken Tumor I, while Tanzer[11] found that testis extract inhibited the growth of mouse Sarcoma 180, but was without effect on Sarcoma 37 and the Bashford Adenocarcinoma. Tanzer suggests that the testis extract may either injure the cells of the tumor, or increase the resistance of the inoculated animal, and that the Duran-Reynals spreading factor may or may not, be identical with the factor acting upon tumors.

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It is apparent that the experimental data are not consistent, and do not permit general conclusions as to the significance of the spreading factor in the growth mechanism of malignant tumors. In the present work we have investigated the effect of various testis extracts on the growth of a transplanted mouse lymphosarcoma. We have also considered the relationship of the principle affecting the growth of tumors to the Duran-Reynals spreading factor, identified with the enzyme hyaluronidase.

*Material and methods.* The experimental work has been done with mice of the AKm stock. The tumor used was derived from the 9417 strain of lymphatic leukemia, described by Burchenal, Lester, Riley and Rhoads.[12] This leukemia is of spontaneous origin. According to Willis[13] we have used the term "lymphosarcoma" for the localized growth of the disease. The tumor was propagated by subcutaneous implants into the right side of the abdomen of the mice. A piece of tumor was minced with scissors in physiological saline solution, filtered through cotton, and the cell suspension diluted to contain 1 million cells in 0.1 ml.

In the experimental animals 0.1 ml (1 million cells) of the tumor suspension was mixed with 0.1 ml of testis extract and inoculated at once into the mice. The control animals were given 0.1 ml of the suspension of malignant cells mixed with 0.1 ml of physiological saline solution. In the control animals a tumor developed at the site of injection after 8-12 days. Successively it attained the size of 3-4 cm in diameter, infiltrated the abdominal musculature and peritoneum, became necrotic, hemorrhagic and finally ulcerated. The homolateral inguinal and axillary lymph nodes were aways involved. The disease usually spread to the internal organs before the animals died on an average of 30 days following the inoculation.

We have used 3 preparations of testis extract from AKm mice designated "crude extract", "granule fraction", and "supernate fraction". All of these represented a concentration of 50% in relation to the amount of testis tissue employed. The "crude extract" was prepared by grinding the testis tissue with equal amounts of distilled water and centrifuging once for 20 minutes at 600 x g. The resulting supernate constituted the "crude extract." The "granule fractions" were prepared by grinding testis tissue with equal amounts of either distilled water or 30% sucrose solution. The homogenates were centrifuged 3 times for 20 minutes at 600 x g and the sediments discarded. The supernates were then centrifuged 30 min. at 20,000 x g, and the sediments consisting of granules staining with Janus Green B, washed once or twice in either distilled water or 30% sucrose solution. After the final centrifugation at 20,000 x g, the sediments were taken up in distilled water, and the fractions designated "granule, water" and "granule, sucrose". Because of the observation of Hogeboom, Schneider and Pallade[14] that the large granules of the cytoplasm (mitochondria) were preserved best in 30% sucrose solution, we found it necessary to compare the activity of the "granules" prepared in distilled water with those prepared in sucrose solution. Since it was not considered desirable to use hypertonic sucrose solution for injection purposes, we used only distilled water for the final suspension. The "supernate fraction" represented the supernate from the first high speed centrifugation of a "granule fraction" prepared with distilled water. This supernate was purified by one or two more centrifugations at high speed.

In preliminary experiments we have investigated the content in the testis extracts of the factor, supposed to be hyaluronidase,

TABLE I.
Showing the Effects of Various Testis Extracts on the Intradermal Spread of India Ink.

| Extracts | Spreading coefficient |
|---|---|
| Crude extract | 21 |
| Supernate fraction | 21 |
| Granule, water | 3 |
| Granule, sucrose | 3 |
| Control | 1 |

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TABLE II.
Showing the Growth of the Lymphosarcoma as Influenced by Testis Extract.

| Inoculum | Total No. | No. with tumor | No. without tumor |
|---|---|---|---|
| Lymphosarcoma + testis extr. | 99 | 55 (55.5%) | 44 (44.5%) |
| Lymphosarcoma + physiol. saline | 43 | 39 (91%) | 4 (9%) |

TABLE III.
Showing the Relative Effect of Different Testis Fractions on Growth of the Lymphosarcoma.

| Inoculated with lymphosarcoma + | Total No. | No. with tumor | No. without tumor |
|---|---|---|---|
| Crude extr. | 39 | 18 (46%) | 21 (54%) |
| Granule fraction | 32 | 19 (59%) | 13 (41%) |
| Supernate " | 28 | 18 (64%) | 10 (36%) |
| Physiological saline | 43 | 39 (91%) | 4 (9%) |

which affects the spread of India Ink injected intradermally. Testis extracts were combined with equal amounts of India Ink, diluted 1:5 with distilled water, and 0.4 ml of the mixtures injected intradermally on the shaved back of albino rabbits. India ink plus distilled water served as control. After 24 hours the longitudinal (D) and latitudinal (d) extent of the spread were measured and the area of spreading estimated according to the formula $\pi \frac{D \times d}{4}$.[15] The figure representing the spread of the extract, divided by that of the control, was designated the spreading coefficient.

*Results.* Table I shows the spread of the India Ink as influenced by the various fractions of testis extract.

As shown in Table I both the "crude extract" and the "supernate fraction" gave very marked spreading effects. On the other hand the "granule fraction" produced a spread of India Ink which was small and hardly significant. It is emphasized that the "granules" gave the same spread, whether they were prepared with distilled water or with 30% sucrose solution.

Tables II and III present the effect of testis extract on the transplanted lymphosarcoma in 99 mice. There were 43 controls. The whole group of animals given testis extract is presented without regard to the particular fractions of the extract in Table II, while Table III relates the effects of the inividual fractions.

In the control group 9% of the animals do not develop tumors, whereas, in the group treated with testis extract, 44.5% fail to take the transplant. This difference is statistically significant. Some of the test animals were killed when the controls died. On autopsy slightly enlarged subcutaneous lymph nodes were found on the same side as the injections. The spleens were small, and there were no macroscopical signs of metastasis to other inner organs. Most of the animals, however, were allowed to live 2 months or more after the inoculation, and appeared to remain in good health.

Table III indicates the relative effect of the "granule" and "supernate" fractions of testis extract on the growth of the lymphosarcoma.

The "crude extract" showed slightly greater activity than the more purified fractions. Interestingly the "granule" and "supernate" fractions seemed to have practically the same activity, with a slight difference in favor of the "granule fraction". The "granules" showed the same effect whether they were prepared with 30% sucrose or distilled water.

If the principle affecting the growth of this lymphosarcoma was identical with the spreading factor (hyaluronidase), we would expect to demonstrate a significant difference between the activity of the "granule" and "supernate" fractions, a difference comparable to that demonstrated in the spreading coefficients (Table I). As shown there the spreading coefficient of the "supernate" is 7 times that of the "granule fraction". In contrast the tumor influencing activity in the "granule fraction" is slightly higher than that of the "supernate fraction". (Table III). These findings indicate that the "lymphosarcoma factor" is not identical with the spreading factor (hyaluronidase).

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[15] Madinaveitia, J., *Biochem. J.*, 1938, **32**, 1806.

*Summary.* A factor has been demonstrated in testis extract which inhibits the growth of a transplanted lymphosarcoma in AKm mice. This factor is of unknown nature, but appears to be different from the Duran-Reynals spreading factor.

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## 17158. Effects of Urethane on Living Tissue.*

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Ethyl Carbamate (urethane) has been shown to be a useful agent in the treatment of wound infections.[1,2] When extensive wounds or large areas of intact skin are exposed to the prolonged application of 10% urethane solution[2] about 20 to 30% of the patients treated develop nausea and vomiting. This suggests that the drug is absorbed through open wounds and normal skin surfaces. The nausea and vomiting reported by Howe and Weinstein.[2] and Howe[1] in urethane treated wound infections and by Paterson *et al.* in leukemia patients receiving the drug by mouth[3] appeared to be benign in nature. However, because the treatment of human wound infections requires prolonged exposure to large amounts of this drug, experiments were conducted to investigate its effect on the organs of animals treated with similar doses.

A group of 10 guinea pigs was given lethal amounts of urethane administered intraperitoneally in rapidly ascending doses over a short period of time (Table I, Pigs 1-10). The gross and histological tissue changes were compared with those of a second group of 10 animals receiving daily subcutaneous injections of urethane in dosages simulating those used in the topical treatment of infected wounds in man (Table II, Pigs 14-23). Hypnotic effects ranging from drowsiness and ataxia to terminal anesthesia were noted. Some animals recovered and ate heartily after 8 hours of complete anesthesia. In the treatment of a large infected wound a person is exposed to a maximum of 60 g of urethane per day, although only a fraction of this is absorbed due to loss in dressings. The animals in Group II received 0.1 g per 100 grams of body weight per day for 28 consecutive days which is about twice as long as the average duration of therapy in 39 urethane treated patients.[2] This is comparable to the maximal dosage used in man, and it was given subcutaneously to insure complete absorption. Dosages were corrected weekly for fluctuations in weight. Control animals (Table I, Pigs 11-13; Table II, Pigs 24-26) received corresponding amounts by volume of normal saline. Diet consisted of Purina chow, lettuce and cabbage. All animals in Group II gained weight and at the end of the experiment were healthy and had lustrous silky coats. They were killed on the 29th day.

Examination of the organs was made immediately after death. Sections were fixed in 5% formaldehyde solution, imbedded in paraffin and stained with hematoxylin eosin. Brain, striated muscle, myocardium, lungs,

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* This study was aided by the President's Fellowship of Brown University.

[1] Howe, C. W., *Surg., Gynec. and Obst.*, 1948, **87**, 425.

[2] Howe, C. W., and Weinstein, L., *Surg., Gynec. and Obst.*, 1947, **84**, 913.

[3] Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

TABLE I.
10% Urethane Intraperitoneally in Lethal Doses.

| Pig No. | Wt in g | Days treated | No. inj. | Total dose, g | Fate |
|---|---|---|---|---|---|
| 1 | 248 | 9 | 5 | 0.99 | Died |
| 2 | 250 | 13 | 6 | 1.32 | ’’ |
| 3 | 316 | 13 | 6 | 1.47 | ’’ |
| 4 | 267 | 9 | 5 | 1.02 | Killed |
| 5 | 269 | 6 | 4 | 0.83 | ’’ |
| 6 | 842 | 3 | 3 | 7.57 | Died |
| 7 | 821 | 3 | 3 | 7.81 | ’’ |
| 8 | 906 | 3 | 3 | 8.19 | ’’ |
| 9 | 757 | 3 | 3 | 6.80 | ’’ |
| 10 | 842 | 3 | 3 | 7.58 | ’’ |
| 11* | 757 | 3 | 3 | 0.58† | Killed |
| 12* | 250 | 10 | 6 | 0.11† | ’’ |
| 13* | 270 | 9 | 5 | 0.09† | ’’ |

* Saline treated control.
† Grams of NaCl.

TABLE II.
10% Urethane Subcutaneously.

| Pig No. | Initial wt, g | Total No. daily inj. | Total dose, g | Final wt, g |
|---|---|---|---|---|
| 14 | 442.0 | 28 | 13.81 | 581.6 |
| 15 | 353.2 | 20* | 8.19 | 468.4 |
| 16 | 396.7 | 20* | 8.46 | 393.4 |
| 17 | 344.5 | 28 | 10.47 | 432.1 |
| 18 | 412.9 | 28 | 13.23 | 528.3 |
| 19 | 399.0 | 28 | 12.44 | 492.4 |
| 20 | 355.5 | 28 | 10.96 | 412.2 |
| 21 | 391.2 | 28 | 12.19 | 503.7 |
| 22 | 441.5 | 28 | 13.82 | 556.6 |
| 23 | 274.1 | 28 | 9.67 | 440.5 |
| 24† | 434.4 | 28 | 1.18‡ | 563.7 |
| 25† | 392.4 | 28 | 1.10‡ | 558.1 |
| 26† | 390.4 | 28 | 1.18‡ | 514.1 |

* Injections omitted after 20th day due to development of pneumonia. Animal recovered.
† Saline treated control.
‡ Grams of NaCl.

liver, spleen, pancreas, kidneys, adrenals and gonads were examined.

*Findings.* Only the significant changes are described. In the group of animals submitted to intraperitoneal injections of lethal doses of urethane (Group II) there was congestion in the lungs, liver and spleen and occasionally in the kidneys. Vascular changes characterized by endothelial swelling and desquamation, mural edema and extravasation of plasma and formed blood elements into the surrounding tissues were occasionally present. Cytoplasmic vacuolization was observed in the hepatic cells and glandular epithelium of the renal tubules. A striking hyperplasia of mesenchymal cells was noted in the lungs and spleen and to a lesser degree in the myocardium and liver. Frequent hepatic cell changes consisting of an increase in fat droplets, cytoplasmic swelling and rarefaction were present. The central lobular cells in 3 animals[3,8,10] displayed loss of cellular outlines. The kidneys revealed no glomerular damage. There were hemorrhages in the cortex and medulla in animals 2 and 9.

In the animals subjected to sublethal injections of urethane over long periods of time (Group II), the striking feature was a mesenchymal cell reaction in the lungs and spleen. Cytoplasmic vacuolization of the hepatic cells was frequently seen. Vascular engorgement in the lungs, liver and spleen often accompanied by mural edema and by escape of plasma and formed blood elements were occasionally encountered.

The kidneys showed no significant findings.

The tissues at the site of injection revealed blood pigment and recently extravasted blood cells in the subcutaneous tissues. Independently from these hemorrhagic changes, a mesenchymal reaction consisting of histiocytes and lymphocytoid cells was found in a number of guinea pigs.

Microscopic sections of the lungs in two control animals showed engorgement of blood vessels, mural edema, minimal endothelial damage and red cell extravasations. In the same two animals there were dilated hepatic sinusoids with red cell extravasations and increased cellularity in the pulp substance of the spleen.

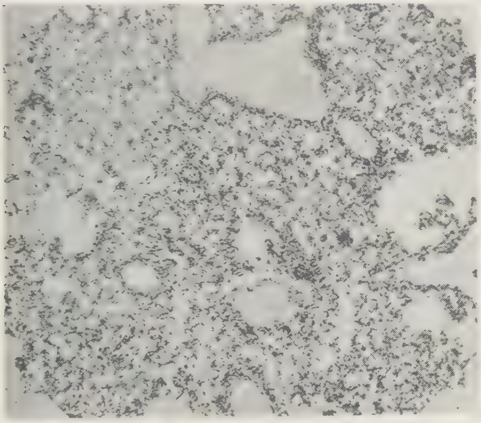

FIG. 1.
Diffuse septal mesenchymal cell infiltration in lung of a guinea pig; × 25.

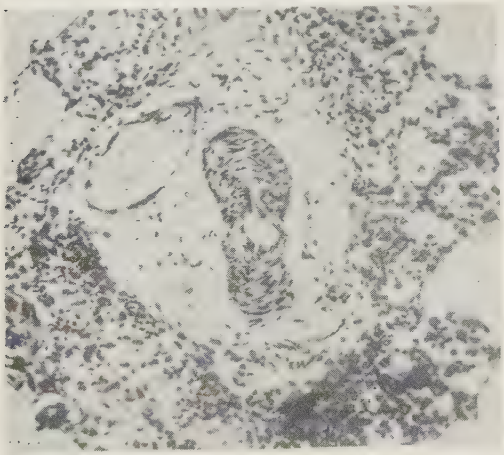

Fig. 2.
Pulmonary artery and vein displaying perivascular edema and mesenchymal reaction; × 125.

*Discussion.* The changes in Group I animals which died from lethal doses of urethane were not significantly greater than those in Group II which were killed in apparent health, to explain the death of the former on a morphologic basis. It is our opinion that death occurred through disturance of the respiratory mechanism as a result of the central hypnotic action of the drug and that the tissue changes in Group II were compatible with healthy life.

Vascular dilation and engorgement in the lungs, liver and spleen at times accompanied by mural changes were consistently seen. The frequent finding of areas of perivascular edema or hemorrhage without evidence of damage to vessel walls might indicate a reversibility of these mural changes. Vascular injury comparable to the mildest forms seen in the urethane treated animals were also observed in two of the saline treated control animals. Although urethane induced renal damage has been reported in specific strains of mice,[4,5] the glomerular apparatus in our guinea pigs remained essentially intact.

The hepatic changes compare with those previously reported by Doljanski and Rosin[6] in a study of urethane treated rats. The cellular damage consisted mainly of cytoplasmic vacuolization, a condition met with under a variety of pathological states. This condition has been produced by perfusion with 0.6 NaCl Solution[7] and Ringer's Solution[8] and vacuolization has been noted as early as 10 minutes after perfusion started. Raum[7] considered it a reversible phenomenon.

A striking reaction of cellular mesenchyma was consistently found in both groups of animals, most apparent in the lungs and spleen. A similar proliferation of mesenchy-

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[4] Dunn, T. B., and Larsen, C. D., *Fed. Proc.*, Part II, 1946, **5**, 220.

[5] Kirschbaum, A., and Bell, E. T., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 71.

[6] Doljanski, L., and Rosin, A., *Am. J. Path.*, 1944, **20**, 945.

[7] Raum, J., *Arch. f. Exp. Path. u. Pharmakol.*, 1892, **29**, 353.

[8] von Skromlik, E., and Hunnerman, T., *Z. f. d. ges. exp. Med.*, 1920, **11**, 349.

mal cells is mentioned by Nettleship *et al.*[9] in urethane treated mice. Paterson *et al.*[3] reported fibrosis in the bone marrow, liver and spleen of urethane treated leukemia patients and the authors have noted excess fibroplasia in excised sections of urethane treated wounds.

It is conceivable that the induction of a degree of vascular dilation and excess fibroblastic proliferation would be beneficial in the treatment of healing wounds.

Although the evidence is insufficient to exclude a direct action of urethane on liver cells, it was productive of comparatively slight hepatic and renal damage even when given in lethal doses. These changes are apparently causally dependent upon vascular injury which is reversible.

*Conclusions.* (1) Doses of urethane simulating those used in the topical treatment of infected wounds in man are not productive of toxic parenchymal changes in the organs of guinea pigs subjected to prolonged daily subcutaneous injections. The animals remained healthy and gained weight.

(2) A marked mesenchymal cell reaction was consistently seen in the lungs and to a lesser extent in the liver and myocardium of urethane treated animals.

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[9] Nettleship, A., Henshaw, P. S., and Meyer, H. L., *J. Nat. Cancer Inst.*, 1943, **4**, 309.

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## 17159. Anticonvulsant Action of 2-Substituted-1,3-Propanediols.

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3-(o-Toloxy)-1,2-propanediol, called myanesin, possesses two important pharmacological properties: it causes transient paralysis of skeletal muscles and has an anticonvulsant action.[1] The pharmacological examination of numerous related ethers of glycerol has shown that there was no simple relationship between their paralysing and anticonvulsant activities. The observation that nuclear substitution of 3-phenoxy-1,2-propanediol markedly affected paralysing activity but did not have any influence on anticonvulsant action[2] suggested the examination of the anticonvulsant properties of 2-substituted-1,3-propanediols.†

The anticonvulsant action of the compounds was determined in male, white mice, weighing 18 to 22 g. Metrazol 100 mg per kg, which caused convulsions and deaths in 99% of the animals, was injected intraperitoneally together with graded doses of the compound under test. Ten mice were used at each dose level. The animals were observed for 90 minutes following the injection and the incidence of convulsions and deaths noted. The relative efficacy of the substances was evaluated by finding graphically the dose protecting one half of the animals from convulsions and deaths. This mean protective dose of 13 experimental compounds and of phenobarbital and myanesin is given in Table I. Several of the investigated substances had anticonvulsant properties of a similar order as myanesin (No. 3, 6, 7, 9). Compounds containing butyl radicals (No. 4 and 8) were inactive. When a phenyl or phenoxy group was a substituent, activity was decreased or lost (No. 11, 12, 13). 2,2-Diethyl-1,3-propanediol, called DEP, possessed outstanding anticonvulsant properties. A short description of its pharmacological properties appears to be of interest.

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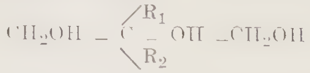
* Present address: Wallace Laboratories, Inc., New Brunswick, N. J.

[1] Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.

[2] Berger, F. M., *J. Pharm. and Exp. Therap.*, 1948, **93**, 470.

† I am obliged to Mr. W. A. Lott of the Squibb Institute for Medical Research for supplying me with some of these compounds.

TABLE I.
Antagonistic Action of 2-substituted-1,3-propanediols to the Lethal Effects of Metrazol 100 mg per kg. Metrazol and one of the other drugs were given simultaneously by intraperitoneal injection to white mice.



$$CH_2OH - \overset{\diagup R_1}{\underset{\diagdown R_2}{C}} - OH - CH_2OH$$

| No. | $R_1$ | $R_2$ | Mean protective dose in mg per kg |
|---|---|---|---|
| 1 | methyl | methyl | >250 |
| 2 | ethyl | ethyl | 40 |
| 3 | n-propyl | n-propyl | 88 |
| 4 | n-butyl | n-butyl | >250 |
| 5 | methyl | ethyl | 135 |
| 6 | ’’ | n-propyl | 70 |
| 7 | ’’ | iso-propyl | 88 |
| 8 | ’’ | n-butyl | 210 |
| 9 | ethyl | iso-propyl | 77 |
| 10 | ’’ | ethoxy | >250 |
| 11 | phenyl | H | >250 |
| 12 | ’’ | ethyl | 120 |
| 13 | phenoxy | ’’ | >250 |
| 14 | phenobarbital | | 39 |
| 15 | myanesin | | 80 |

DEP resembled myanesin in causing transient paralysis of skeletal muscles. The paralysing activity of DEP was, however, of a lower order than that of myanesin, the doses causing loss of the righting reflex in 50% of mice after intraperitoneal administration being 390 mg per kg and 180 mg per kg, respectively. In small doses not causing paralysis DEP somewhat increased the spontaneous activity of the animals whereas myanesin had a sedative effect. The acute toxicity of DEP was very low. The mean lethal dose in mice on intraperitoneal administration was 1400 mg per kg. Doses as high as 30 mg per kg given intravenously to anesthetized cats did not affect blood pressure and respiration. Similar doses did not alter normal electroencephalographic patterns in cats and did not influence spinal reflexes. On a weight for weight basis DEP was as active as phenobarbital in preventing the occurrence of convulsions and deaths from lethal doses of metrazol. It was more effective than myanesin, phenobarbital or other drugs in antagonizing the lethal and convulsant effects of strychnine.

DEP was more effective against convulsions induced by chemical agents than against those induced by electric current. It abolished the tonic extensor component of supramaximal electroshock seizures in mice and rabbits but was in this respect about 6 times weaker than phenobarbital. The duration of the effect was also much shorter with DEP than with phenobarbital.

Myanesin is known to abolish abnormal electroencephalographic patterns in cases of true petit mal.[3] Because of the qualitatively similar anticonvulsant effect of DEP and myanesin, the stronger anticonvulsant action of DEP may be of value in the treatment of petit mal and of other disorders in which myanesin has been tried.

*Summary.* 2,2-Diethyl-1,3-propanediol and certain other substituted 1,3-propanediols possess qualitatively similar pharmacological properties as myanesin. These compounds differ from myanesin in having a weaker paralysing action and a more powerful anticonvulsant action.

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[3] Gammon, G. D., and Churchill, J. A., *Am. J. Med. Sci.*, 1949, **217**, 143.

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## 17160. The Golden Hamster (*Cricetus auratus*) as an Experimental Animal for Poliomyelitis Research.*

MAX R. STEBBINS AND SERGE G. LENSEN. (Introduced by C. W. Muehlberger.)
*From the Division of Laboratories, Michigan Department of Health, Lansing, Mich.*

Successful passage of the Lansing strain of poliomyelitis virus to the Syrian or golden hamster was reported by Armstrong and Packchanian,[1] Plotz, Reagan and Hamilton,[2] and Durand.[3] The purpose of this study was to investigate how the golden hamster (*Cricetus auratus*) compares to the white mouse and the cotton rat as a laboratory animal for experimental work with the Lansing strain virus.

*Material and methods. Virus.* Suspensions of spinal cords and medullae of infected animals were made up in buffered saline (pH 7.2-7.4). Hamster-passage, mouse-passage and cotton-rat-passage virus was used.

*Hamsters.* Two strains of golden hamsters were used, one bred at the Michigan Department of Health (designated here as MDH-hamsters), the other obtained from the Tumblebrook Farm, Brant Lake, N. Y. (referred to as TF-hamsters).

*Comparative titrations and neutralization tests.* Five 5-fold dilutions were used in the comparative titrations. The numbers of animals for each dilution were: 4 for cotton rats and hamsters, and 6 for mice. All the neutralization tests were carried out in mice using constant amounts of mouse-passage virus and decreasing amounts of serum. The animals were observed for a period of 30 days in all the experiments.

*Titers and inocula.* The $LD_{50}$ titers of the virus preparations were calculated by the method of Reed and Muench[4] and expressed in terms of dilution of the original tissue material (in %).

*Experimental. Serial passages.* Serial passages were carried out in MDH-hamsters as well as in TF-hamsters. Mouse-passage virus was used for the initial passage, followed by hamster-to-hamster passages. All the passages were done by intracerebral inoculation of 10% suspensions of cords and medullae.

As shown in Table I, the outcome of hamster-to-hamster passages did not show considerable differences between the 2 strains of hamsters, either in the percentage of takes or in the average incubation period. In the sixth passage in the MDH-hamsters none of the animals developed any symptoms. No attempt was made to continue the passages in TF-hamsters beyond the sixth passage in view of the low percentage of takes.

*Comparative virus titrations.* The outcome of the titrations of mouse passage, cotton-rat passage and hamster passage virus in mice, cotton rats and hamsters is summarized in Table II. The data presented show that the mouse passage and the cotton-rat passage virus gave extremely low $LD_{50}$ titers in MDH-hamsters (5% and more), while the same virus preparations gave high titers in cotton rats and mice (ranging from .028 to .0008% dilutions of virus). The MDH-hamster passage virus gave extremely low titers in MDH-hamsters (over 10%) as well as in cotton rats (5%) and mice (1.67%).

Considerably better results were obtained with TF-hamsters. Both mouse passage and cotton rat passage virus gave good titers in TF-hamsters (.015 and .09% respectively). The TF-hamster passage virus gave the lowest titer in TF-hamsters (.6%), but this titer was considerably higher than the corresponding titer obtained in MDH-hamsters (more than 10%). The general pattern was, however, the same with the 2 strains of hamsters: the use of the hamster as an experimental

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* The data presented in this paper were used by the senior author for a thesis in partial fulfillment of the requirements for the Master of Science degree at the Michigan State College.

[1] Armstrong, C., and Packchanian (unpublished data, see Vanderbilt Lectures, 1941).

[2] Plotz, H., Reagan, R., and Hamilton, H. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 124.

[3] Durand, P., *Compt. Rend. Soc. Biol.*, 1943, **139**, 716.

[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Serial Passages of Lansing Strain Virus in Hamsters.

| | No. of animals inoculated | | No. of animals paralyzed | | Incubation period in days | |
|---|---|---|---|---|---|---|
| Passage No. | MDH | TF | MDH | TF | MDH | TF |
| 1 | 4 | 4 | 2 | 4 | 3, 4 | 2, 3, 3, 5 |
| 2 | 5 | 4 | 2 | 3 | 3, 11 | 3, 3, 18 |
| 3 | 5 | 4 | 1 | 2 | 3, | 4, 4 |
| 4 | 5 | 4 | 4 | 1 | 4, 4, 8, 9 | 4 |
| 5 | 4 | 4 | 1 | 2 | 8 | 5, 16 |
| 6 | 5 | 4 | 0 | 1 | — | 6 |
| | | | | | Avg 5.7 | Avg 5.1 |

TABLE II.
Comparative Titrations.

| Virus | Titrated in | $LD_{50}$ (in %) |
|---|---|---|
| MDH-hamster passage virus | MDH-hamsters | >10.0 |
| | Cotton rats | 5.0 |
| | Mice | 1.67 |
| TF-hamster passage virus | TF-hamsters | .6 |
| | Cotton rats | .018 |
| | Mice | .054 |
| Mouse-passage virus | MDH-hamsters | 5.0 |
| | TF-hamsters | .015 |
| | Cotton rats | .004-.0008 |
| | Mice | .0016-.0008 |
| Cotton rat passage virus | MDH-hamsters | >5.0 |
| | TF-hamsters | .09 |
| | Cotton rats | .029-.009 |
| | Mice | .028-.0015 |

animal, or of hamster passage virus, resulted in lower titers than when mice or cotton rats were used, and the lowest titers were obtained when hamster passage virus was titrated in hamsters.

*Attempts to increase the infectivity of the Lansing strain for the golden hamster.* Attempts were made to enhance the infectivity of the Lansing strain virus for TF-hamsters by using as diluent saline buffered at a low pH (technique of Hammon and Izumi[5]). In one experiment (with cotton rat passage virus) the result was better at pH 4, while in 2 other experiments, for which mouse-passage virus was used, better results were obtained at pH 7.2.

In 2 other experiments with TF-hamsters the technic described by Milzer and Byrd[6] (autolyzed normal mouse brain tissue as adjuvant) was used. No enhancement of the infectivity was observed.

*Immune response.* It seemed interesting to investigate the immune response of the golden hamster to the Lansing strain virus. For this purpose, hamsters were immunized by intraperitoneal inoculations of .3 ml of a 10% suspension of live virus. MDH-hamsters were immunized with the mouse passage virus, while mouse passage, cotton rat passage and hamster passage virus were used for the immunization of 3 separate lots of TF-hamsters. The vaccinations were done twice weekly with a total of 7 vaccinations. In each lot, one group of animals was bled 2 weeks after the first vaccination, another group after 3 weeks, and still another group after 4 weeks (6 to 10 days after the last vaccination). The sera of each group were pooled and are referred to as 1st, 2nd and 3rd bleeding, respectively. The antibody content was determined by neutralization tests in mice.

TF-hamsters vaccinated with TF-hamster passage virus showed the weakest immune response with the maximum titer of 1:27. The titers of the sera of TF-hamsters vaccinated with mouse passage virus were 1:20 in the first bleeding, 1:47 in the second and 1:45 in the third bleeding. The vaccination of the MDH-hamsters with mouse passage virus gave a similar titer in the first bleeding (1:24) and a higher titer in the second bleeding (better than 1:96). There was a drop of the titer in the third bleeding.

The best results were obtained in TF-hamsters vaccinated with cotton rat passage virus. The titer was 1:17 in the first, better than 1:73 in the second, and better than 1:126 in the third bleeding.

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[5] Hammon, W. McD., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

[6] Milzer, A., and Byrd, C. L., *Science*, 1947, **105**, 70.

*Discussion.* The two strains of golden hamsters used by the authors differ considerably in their susceptibility to the Lansing strain virus and in the titer of the hamster passage virus. One of the strains (MDH) is clearly not suitable for experimental research with the Lansing strain virus, and the other (TF) which gave considerably more favorable results, also is in every respect inferior to both the white mouse and the cotton rat.

The immunization of the hamster with active Lansing strain virus resulted in fairly good titers of neutralizing antibody, provided cotton-rat passage or mouse passage virus was used for immunization. In view of the small size of the hamster, use of this animal for serological work with the Lansing strain virus does not seem to present any particular advantage.

*Summary and conclusions.* Golden hamsters (*Cricetus auratus*) from two different sources were found to present marked differences in their susceptibility to the Lansing strain of poliomyelitis virus. Both strains were found to be markedly inferior to the white mouse and to the cotton rat as experimental animals for poliomyelitis research.

The adjustment of the inoculum at a low pH did not increase the titer of the hamster passage virus consistently. The use of the autolyzed normal mouse brain technic did not increase the infectivity of the virus for the hamster.

The intraperitoneal inoculations of mouse-passage and cotton-rat passage virus resulted in higher titers of neutralizing antibody in the golden hamster than when hamster passage virus was used.

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## 17161. Relation of Pregnancies to Induction of Ovarian Tumors by X-rays.

Jacob Furth.

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Two forces are now postulated in the genesis of ovarian tumors: direct delayed x-ray effect and a hormonal "imbalance." Ova are highly sensitive to x-rays. Their destruction disrupts the normal development of the ovarian follicles. It is believed that new formation of ova occurs in young female mice, therefore, it can be assumed that the hormonal imbalance induced by x-rays in very young mice as well as that caused by small doses of x-rays is slight and under such conditions ovarian tumors are less likely to occur. These were the thoughts that led to the pilot experiments here described.

*Irradiation of Mice at 1-3 Days of Age*: Earlier experiments have shown that a single exposure to 87r or more at 5-10 weeks of age will produce tumors in most mice when they reach adult age.[1]

In the first experiment reported here, mice were irradiated with 150r at 1-3 days of age and were reared by their non-irradiated mothers and allowed to live until natural death or about 20 months of age. At about 4-6 weeks of age they were weaned and a few weeks later mated with normal males. When pregnant they were separated, allowed to nurse their babies after which they were returned to the mating cage. Table Ia indicates that about 30% of these mice became pregnant and some had as many as 4 pregnancies. Ovarian tumors developed in 76%. One-half of those that were pregnant remained free from ovarian tumors as compared to 8% of those that were completely sterilized by x-rays. However, even multiple pregnancies failed to prevent the development of ovarian tumors. The time of appearance of these neoplasms is not significantly shorter than that in mice irradiated at 4-6 weeks of age.[1]

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[1] Furth, J., and Boon, M. C., *Cancer Research*, 1947, **7**, 241.

TABLE Ia.
Effect of Irradiation of 1-3-Day-Old Mice (Rf/Ak) with 150r.

| Length of life (mo.) | No. in group | No. pregnant | No. of pregnancies 1 | 2 | 3 | 4 | Neoplasms Ovary | Lung | Leukemia |
|---|---|---|---|---|---|---|---|---|---|
| 7 | 2 | 1 | 1 | | | | | | |
| 8-13 | 5 | 2 | 2 | | | | 2 | 1 | |
| 14-18 | 13 | 6 | 3 | 2 | 1 | | 9 | 3 | 3 |
| 19-23 | 39 | 9 | 3 | 2 | 2 | 2 | 34 | 15 | 1 |
| Total | 59 | 18 | 9 | 4 | 3 | 2 | 45 | 19 | 4 |
| % | | 30.5% | | | | | 76.3% | 32.2% | 6.8% |

Factors of irradiation were as follows: 140 kv., 5 m. amp., 25 cm target skin distance, with an inherent filtration of 1 mm of aluminum, machine delivering 120r per minute.

TABLE Ib.
Controls.

| Length of life (mo.) | No. in group | Neoplasms Ovary | Lung | Leukemia |
|---|---|---|---|---|
| 7 | 1 | 0 | 0 | 0 |
| 8-13 | 2 | 0 | 0 | 0 |
| 14-18 | 7 | 0 | 1 | 0 |
| 19-22 | 28 | 0 | 9 | 1 |
| Total | 38 | 0 | 10 | 1 |
| % | | | (26.3%) | (2.6%) |

A group of 38 closely related mice were set aside as controls for the incidence of ovarian and other tumors. No pregnancy records were kept on these mice but it may be assumed that they were fertile. None developed ovarian tumors as shown in Table Ib.

While this slight increase in the incidence of leukemia and lung tumors in x-rayed mice may not be significant, the figures are in line with those observed in earlier larger experiments.[2-5]

The brothers of the female mice irradiated at 1-3 days of age with 150r were also observed until natural death or about 20 months of age. Only one developed a testicular interstitial cell tumor.

Ten Ak mice received 150r at the advanced stage of pregnancy. The females born 1 to 5 days after irradiation were autopsied at 7 to 15 months of age. None had grossly detectable ovarian tumors. Three examined microscopically have shown changes as those seen in x-rayed animals.

*Irradiation of Mice with 25-200r at 5 Weeks*: In these experiments C3H mice obtained from the L.C. Strong were used. All other stocks of mice tested earlier were highly susceptible to the induction of ovarian tumors and it was thought that the use of C3H mice susceptible to breast cancers might disclose additional facts.

Table II shows that with increase of the irradiating dose the number of pregnancies is reduced while the ovarian tumor incidence is increased. The only figure out of line of this statement is the lower percentage of ovarian tumors in mice irradiated with 100r. Loss from intercurrent disease was high among these mice. The overall low tumor incidence and the above irregularity may be due in part to poor health and early death of many mice.

A summary analysis of all pertinent data is in Table III.

*Comments.* The data here recorded indicate that mice x-rayed at 1-3 days or 5 weeks of age can have one or several normal pregnancies although they are destined to develop ovarian tumors at a later age. The data do not answer the question which group is more liable to develop ovarian tumors, the x-rayed sterile or x-rayed fertile group. Should induction of ovarian tumors be solely due to a hormonal "imbalance" produced by x-rays, one would expect a much lower incidence of ovarian tumors in mice that go through normal

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[2] Furth, J., and Furth, O. B., *Am. J. Cancer*, 1936, **28**, 54.

[3] Kaplan, H. S., *Cancer Research*, 1947, **7**, 141.

[4] Lorenz, E., Heston, W. E., Eschenbrenner, A. B., and Deringer, M. K., *Radiology*, 1947, **49**, 274.

[5] Henshaw, P. S., Riley, E. F., and Stapleton, G. E., *Radiology*, 1947, **49**, 349.

TABLE II.
Effect of Irradiation of C3H Mice at 5 Weeks of Age.

| Dose | Length of life (mo.) | No. in group | No. of mice pregnant | No. of pregnancies 1 | 2 | 3 | Neoplasms Ovary | Lung | Leukemia |
|---|---|---|---|---|---|---|---|---|---|
| 25r | 7 | 2 | 2 | 1 | 1 | | | 1 | |
| | 8-13 | 7 | 7 | 5 | 2 | | | 1 | |
| | 14-19 | 8 | 4 | 1 | 1 | 2 | 2 | 1 | 1 |
| | Total | 17 | 13 | 7 | 4 | 2 | 2 | 3 | 1 |
| 50r | 9-13 | 7 | 4 | 2 | 2 | | | | 1 |
| | 14-19 | 13 | 7 | 7 | | | 4 | 2 | |
| | Total | 20 | 11 | 9 | 2 | | 4 | 2 | 1 |
| 100r | 7 | 1 | | | | | | | 1 |
| | 8-13 | 5 | 1 | 1 | | | | 1 | |
| | 14-18 | 7 | 1 | 1 | | | 2 | | |
| | 19-23 | 3 | 1 | 1 | | | 1 | 1 | |
| | Total | 16 | 3 | 3 | | | 3 | 2 | 1 |
| 200r | 9-13 | 16 | 3 | 1 | | | 4 | 1 | 2 |
| | 14-18 | 5 | 2 | 1 | | | 2 | | 1 |
| | 19-23 | 5 | | | | | 4 | 1 | |
| | Total | 26 | 5 | 2 | | | 10 | 2 | 3 |

In addition the following number of mice had borderline or "pretumors";[6] 2 in the 25r; one in the 50r; and 4 in the 200r groups.

TABLE III.
Induction of Ovarian Tumors by Small Doses and Relation to Pregnancy.

| Mice, stock | C3H | | | | | | | | Rf/Ak |
|---|---|---|---|---|---|---|---|---|---|
| Age at irradiation: | | | | | | | 4-6 weeks[7] | | 1-3 days |
| Dose: | 25r | 50r | 100r | 200r | 25-200r* | 50-200r* | 87r | 175r | 150r |
| % x-rayed developing ovarian tumors | 12% | 30% | 19% | 39% | 24% | 31% | 84% | 94% | 76% |
| Sterile mice, No. | 4 | 9 | 13 | 21 | 47 | 43 | | | 41 |
| No. developing ovarian tumors | 2 | 2 | 2 | 7 | 13<br>(27.6%) | 11<br>(25.6%) | | | 37 |
| Pregnant mice, No. | 13 | 11 | 3 | 5 | 32 | 19 | | | 18 |
| No. developing ovarian tumors | 0 | 2 | 1 | 3 | 6<br>(18.7%) | 6<br>(31.6%) | | | 8 |

* Combined.

pregnancies than in those sterilized by x-rays. The immediate hormonal "imbalance" caused by small doses of x-rays is slight if any, yet the liability of these mice to develop ovarian tumors is great. X-rays are powerful mutagens and cause tumors in organs not under hormonal control. It seems that neither the skin nor the hemopoietic tissues wholly recover from even minor damage caused by x-rays.[8] The same seems true for the ovary and it is possible that a chromosomal change is a basic factor in the genesis of neoplasm induced by x-rays. However subsequent hormonal influences may eventuate or suppress the appearance of ovarian neoplasms.[9]

Data on the factors of induction of ovarian tumors by radiation are meager. It is known that they can be induced by very small doses. The present experiments show that the amount of x-rays required to induce ovarian tumors is about 25 to 50r if given in a single dose. In the experiments of Lorenz and associates[4] a single dose of 50r produced tumors in 70% of the mice. Daily exposure to 0.11r (from radium) with an accumulated total dose of 90r produced ovarian tumors in about 70% of the mice.[4] No hematological changes have been detected with a daily exposure of 0.1r of gamma radiation in animals that subsequently developed ovarian tumors.[10] An increase in the ovarian tumor incidence in mice exposed to single doses of 26n to 90n of fast neutrons is on record.[5] This is probably much above the minimal tumor producing dose. If mutation is the basis of tumor induction by x-rays, with an increase in dosage an increase in the number of tumors should be anticipated. The present experiments do not exclude this possibility in the range of about 25 to 200r while earlier experiments in the dose range above 87r[1] and those of Lorenz and associates[4] failed to show such a trend.

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[6] Butterworth, J. S., *Am. J. Cancer*, 1937, **31**, 85.

[7] Earlier experiments cited.[1]

[8] Stone, R. S., *Radiology*, 1947, **49**, 297.

[9] Li, M. H., and Gardner, W. V., *Cancer Research*, 1949, **9**, 35.

The relation of age to tumor induction was studied by Kaplan[3] who irradiated 2 weeks to 6 months old mice with 600r administered in daily doses of 50r on 12 consecutive days. The greatest ovarian tumor incidence occurred in mice irradiated at 1 month. None was noted in 7 mice irradiated at 2 weeks but intercurrent mortality was high in this group. The present studies show that mice are susceptible even at 1-3 days of age.

A follow-up of these pilot experiments is desirable, even though each requires about 3 years. The relation of the dose of x-rays and age of hosts to the rate of ovarian tumor induction requires a closer check. The hormonal state of mice preceding tumor development and the incidence of pregnancies and abortions should be determined more accurately. The histological changes in ovaries following irradiation ante- and postpartum deserve further consideration, particularly the alleged postnatal ovogenesis and the regenerative phase which leads to neoplasms. Does irradiation induce a chromosomal change and what initiates and sustains the hormonal imbalance are questions to be answered.

*Summary.* Mice exposed to 50-200r of x-rays can have one or several normal pregnancies and still be liable to the development of ovarian tumors in a stock in which this neoplasm is practically non-existent.

Irradiation at 1-3 days of life with 150r sterilized only 1/3 of the mice while ovarian neoplasms appeared in about 76%. Irradiation at 1-3 days did not hasten the onset of ovarian neoplasms as compared to irradiation at 4-10 weeks. In both groups the tumors developed in middle aged and old mice. The growth rate of the tumors was likewise slow, hardly interfering with the normal life span of these animals.

It is postulated that a specific delayed x-ray effect coupled with a hormonal imbalance provoked by x-rays leads to the development of ovarian tumors.

These experiments were begun at Cornell University Medical College and assisted by Thelma Weaver Mold.

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[10] Jacobson, L. O., and Marks, E. K., *Radiology*, 1947, **49**, 286.

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## 17162. Correction of Steatorrhea in Bile Fistula Dogs by Frequent Return of Bile.*

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It was previously reported that 3 to 6 g daily doses of various bile acid preparations mixed with a fatty meal were at best only partially effective in correcting the steatorrhea of bile fistula dogs.[1] This was attributed to one or more of the following possibilities: *a*,

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* Supported in part by a grant from G. D. Searle & Co., Chicago, Ill.

[1] Heersma, J. R., and Annegers, J. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 339.

a physiologically inactive bile preparation; *b*, an inadequate total dosage; *c*, improper time of administration. In subsequent studies, *a* and *b* were minimized by returning all of animals' own bile to the intestine instead of giving commercial bile preparations. As to *c*, flow of concentrated gallbladder bile into the intestine during gastric evacuation and digestion of a fatty meal was shown to be unnecessary for normal fat absorption, since no steatorrhea developed in cholecystectomized dogs.[2] The present experiments involving the return of bile fistula dogs' bile at different intervals throughout the day were designed to determine whether bile must be present continuously for normal fat absorption.

*Methods.* Rous-McMaster external bile fistula dogs[3] were prepared with an additional rubber tube for return of bile into the duodenum.[4] Beginning 7 to 10 days after operation, diets containing 335 g of commercial dog food ("Pard") and 25 g of lard (36 g of total fat) were fed once daily. All meals were completely consumed. Total fecal fat excretion was determined[5] on 5-day stool collections when no bile was returned and when the animals' own bile was returned. Cholic acid in the bile returned during 5-day periods was determined[6] on pooled aliquots. The bile collected during 8, 4, or 1-hour intervals was introduced into the duodenum at the rate of 5 cc per minute.

*Results.* When no bile was returned, fecal fat excretion averaged 25.7 g per day in 6 dogs. This value is in agreement with fecal fat excretion of 27.3 g per day obtained in a previous study in 9 cholecystonephrostomized dogs receiving the same diet.[7]

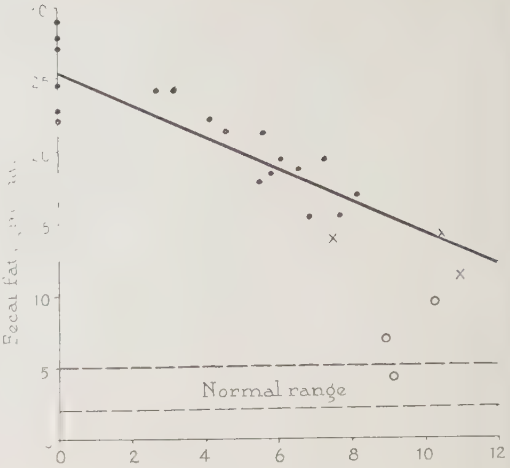

FIG. 1.

Relationship between fecal fat excretion and cholic acid of whole dog bile introduced into the intestine.

● Bile returned every 8 hr
x " " " 4 "
o " " " 1 "

The straight line represents the regression equation fitted to the filled circles only.

In 4 dogs bile was returned every 8 hours for 2 to 10 consecutive 5-day periods. The daily quantity of bile acid secreted and returned increased to reach a steady-state level by the third period, in agreement with the results of Berman *et al.*[4] The average daily quantity of cholic acid secreted and returned during the 5-day tests, and the corresponding daily excretion of fecal fat are plotted for each dog in Fig. 1 (filled circles). As the dose of cholic acid increased, the fecal fat output decreased in linear fashion; the regression equation (correlation coefficient was –0.88) indicated that for each gram increase in cholic acid return fecal fat excretion decreased by 1.17 g. However, at the steady-state level of cholic acid secretion and return (6 to 8 g/day), the defect in the fat absorption was only about 50% corrected. Extrapolation of the regression line implied that 20 g of cholic acid per day would be required for normal fat absorption when the bile is returned every 8 hours. This amount greatly exceeds the steady-state output obtained in this and a previous study,[4] and hence indicates that a time factor as well as a dose factor must be concerned.

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[2] Heersma, J. R., and Annegers, J. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 140.

[3] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.

[4] Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J., *Am. J. Physiol.*, 1941, **131**, 776.

[5] Fowweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

[6] Irvin, J. L., Johnson, C. G., and Kopola, J., *J. Biol. Chem.*, 1944, **153**, 439.

[7] Heersma, J. R., and Annegers, J. H., *Am. J. Physiol.*, 1948, **153**, 143.

Accordingly, bile was returned every hour in 3 dogs for 6 days. Fat absorption during the last 5 days was within normal limits[7] in one dog and considerably improved in the others. Since the plotted points (open circles) are well below the regression line in Fig. 1, the improvement appeared to be due largely to the continuous presence of bile, and not to the increased quantity of cholic acid secreted and returned. To check this point further, bile was returned every 4 hours in the same 3 dogs. The quantity of cholic acid returned did not change, but fecal fat excretion increased so that the plotted values (crosses) fell near the original regression line.

Finally, fecal fat excretion was measured in 3 dogs after cessation of bile return, in order to determine the rapidity with which the fat absorptive defect develops when bile is excluded. From the third through the seventh day after bile return was stopped the fecal fat excretion averaged 17.8 g per day; from the eighth through the thirteenth day, 22.0 g per day. The former value is significantly less, by group comparison analysis,[8] than the mean fecal fat excretion obtained in the present and previous[7] study during 5-day periods begun 10 days or more after the operation. The results indicate that impaired fat absorption develops rapidly when the bile is first excluded, then more slowly to a maximum after about 7 days. The presence of bile acids in the scraped intestinal mucosa of dogs 24 hours, but not 1 month after bile exclusion,[9] suggests a possible reason for the delay in development noted.

*Discussion.* The present results demonstrate that previous failure to correct steatorrhea in bile fistula dogs by feeding them whole ox-bile containing 6 g of cholic acid with the single daily meal was probably due to the failure of this procedure to provide adequate amounts of the bile in the intestine over the period required to complete fat absorption. Although continuous hourly administration of bile was employed successfully in this study to restore normal fat absorption, this does not imply that hourly return must be continued *throughout a 24-hour day* to complete the absorption of a single daily fat meal. However, the establishment of the fact that bile must be present in the intestine continuously for at least most of the day now makes it possible to investigate further the bile component(s) and dosages necessary for normal fat absorption, and to assay bile replacement measures.

*Summary.* The steatorrhea of 3 bile fistula dogs given a fatty meal once daily was nearly completely corrected when the animals' own bile was returned to the duodenum every hour. The steatorrhea persisted, however, when bile was returned every 4 or 8 hours. Since the total quantity of cholic acid returned during the 1- and 4-hour regimes was the same, the importance of continuous presence, apart from total daily quantity present, in the intestine is demonstrated.

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[8] Snedecor, G. W., *Statistical Methods*, Iowa State College Press, 1946.

[9] Verzar, F., and Von Kuthy, A., *Biochem. Z.*, 1931, **230**, 451.

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## 17163. Observations on Experimental Dental Caries. XI. Influence of Pregnancy.

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The results of the clinical investigations on the relationship between pregnancy and dental caries have been contradictory. However, most of the controlled studies made in the last years negate the validity of the old popular idea that pregnancy increases the susceptibility to dental caries. Day,[1] for example, in a recent study carried out with a large number of subjects who had gone through various numbers of pregnancies, and who were divided into several age groups, found no significant differences which could support the statement of a relationship between pregnancy and dental caries incidence. Furthermore, Ziskin and Hotelling,[2] also in clinical studies, found evidence to show that pregnancy not only does not increase dental caries but that factors operating during pregnancy actually prevent tooth decay to a significant degree.

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[1] Day, C. D. M., *Indian J. Med. Research*, 1947, **35**, 101.

In hamsters, we[3] found that pregnancy *and* lactation do not influence caries activity in any direction. Considering Ziskin and Hotelling's findings, we have studied in the present experiment the influence of pregnancy alone, in order to test the possibility that a significant decrease of dental caries activity brought about by pregnancy alone, might be annulled by a harmful effect of lactation.

*Experimental.* Thirty female hamsters, 23 days old, from litters of a colony maintained on Purina Laboratory Chow* and raw milk, were littermate distributed into two groups as follows: Group I, 17 females, and Group II, 13 females. Both groups were reared for 100 days on the following diet of sub-optimal nutritional value: finely ground yellow corn 25%, sucrose 25%, corn starch 20%, powdered whole milk 22%, ether-extracted yeast 5%, alfalfa meal 2%, salt mixture† 1%, and d,l-α-tocopherol acetate 0.010%. The ration and tap water were available *ad libitum.*

Both groups were kept during the first 56 experimental days in screen bottom cages without bedding. At this time the 2 groups were transferred to breeding cages containing sawdust on the bottom, and excelsior for bedding; thereafter 9 sexually mature males kept on the stock colony diet were mated for 13 days with the 17 females of Group I. Every litterborn from Group I was removed from the mother within 6 hours after parturition. Both groups remained in the breeding cages until the end of the experiment.

TABLE I.
Caries Activity in the Two Groups.

| | Group I (pregnant) 14 ♀♀ | Group II (nonpregnant) 13 ♀♀ |
|---|---|---|
| % of animals affected | 100 | 100 |
| Avg No. of carious molars | 8.0 | 8.5 |
| Avg No. of carious lesions | 11.5 | 10.8 |
| Avg caries scores | 6.0 | 6.2 |

On completion of the 100 day experimental period the animals were sacrificed and autopsied. The molars were prepared for examination in the usual way,[4] and the carious lesions were recorded and scored.[5]

*Results.* In group I out of the 17 mated females 2 died during labor, and one had sterile mating. Therefore, only 14 females, those who had normal parturitions, finely formed Group I.

Table I shows the caries activity of the two groups. It is apparent that essentially the same incidence and extent of carious lesions were found in both groups. Therefore, under the conditions of this experiment, pregnancy did not influence in any direction the caries activity. Thus can be stated that neither pregnancy and lactation,[3] nor pregnancy alone influence in any direction the caries susceptibility of the Syrian hamster. These experimental findings agree with the results of most of the controlled clinical studies carried out in the last years[1] on this subject.

It is generally recognized that pregnancy and lactation are physiological states of increased nutritional stress for the mother, since she has to face the double task of supplying proper amounts of nutrients necessary for the offspring as well as her own metabolism. Certain workers[6] have stated that the increased caries activity during pregnancy reported by them has been due to quantitative and/or qualitative deficiencies in the diet. In our previous experiment on the influence of pregnancy and lactation[3] on caries activity, as well as in the present study, we used a sub-optimal diet in order to sharpen the nutritional deficiency of the group undergoing pregnancy, and thus obtain sharper differences of caries activity between the two groups in case of any relationship between dietary deficiencies and dental caries.

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[2] Ziskin, D. E., and Hotelling, H., *J. D. Res.*, 1937, **16**, 507.

[3] Granados, H., Glavind, J., and Dam, H., *Odontologisk Tidskrift*, 1948, **56**, 388.

* From Ralston Purina Co., St. Louis, Mo.

† The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg $CuSO_4$, $5H_2O$, and 556 mg $MnSO_4$, $4H_2O$ per 100 g.

[4] Granados, H., Glavind, J., and Dam, H., *Acta Pathol. et Microbiol. Scandinav.*, 1948, **25**, 453.

[5] Keyes, P. H., *J. D. Res.*, 1944, **23**, 439.

[6] Wohl, M. G., Dietotherapy. Clinical application of modern nutrition, W. B. Saunders Co., Philadelphia, 1945, p. 540.

However, as it can be seen from the data presented, neither pregnancy and lactation[3] nor pregnancy alone influenced caries activity in any direction. These facts are in open contradiction with the alleged increase of caries activity as a consequence of dietary deficiencies during pregnancy.

*Summary.* The influence of pregnancy on caries activity has been studied in hamsters reared from weaning for 100 days on a sub-optimal diet, with their respective littermate controls. The results show that pregnancy does not influence caries activity in any direction.

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## 17164. Evidence for a Steroid Compound in Cane Juice Possessing Antistiffness Activity.

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The isolation from crude cane molasses and crude unheated cane juice of a fraction capable of alleviating an induced wrist stiffness in guinea pigs raised on a skim milk diet, was described by van Wagtendonk and Wulzen.[1] Later investigations by Oleson, *et al.*,[2] and Petering and coworkers[3] indicate that ergostanyl acetate and α-ergostanyl acetate are effective curative agents. We are therefore prompted to report that an active fraction of steroid nature has been isolated from the original cane juice fraction, and in our assay methods has proved to be a curative agent in dosages of at least 0.01 γ.

*Experimental.* A product obtained from the cane juice and melting at 81-82°[1] did not prove to be homogeneous when subjected to carbon-hydrogen analyses. It was therefore subjected to various procedures for further purification. Chromatic adsorption on columns of magnesium oxide or activated alumina failed to yield pure and homogeneous products. Repeated recrystallization from alcohol, followed by recrystallizations from acetone and cyclohexane yielded a compound melting at 164-166° that was homogeneous according to the carbon and hydrogen analyses and highly active in curing the induced stiffness in deficient guinea pigs.

*a. Activity tests.* The assay for activity was carried out according to the previously described methods.[1,4] The compound was dissolved in Wesson oil and administered orally to guinea pigs that had been kept on the deficient regime of skim milk powder to which the necessary vitamins and minerals had

TABLE I.

Therapeutic Test of High Melting Compound Carried Out on Guinea Pigs on Skim Milk Ration.

| Level of assay in γ (5 times) | No. of animals | Cured | No cure |
|---|---|---|---|
| 1 | 3 | 3 | 0 |
| 0.01 | 27 | 23 | 4 |

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* Present address: Argonne National Laboratory, Chicago, Ill.

[1] van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, 1946, **164**, 597.

[2] Oleson, J. J., van Donk, E. C., Bernstein, S., Dorfman, L., and Subbarow, Y., *J. Biol. Chem.*, 1947, **171**, 1.

[3] Petering, H. G., Stubberfield, L., and Delor, R. A., *Arch. Biochem.*, 1948, **18**, 487.

[4] van Wagtendonk, W. J., and Wulzen, E., *Arch. Biochem.*, 1943, **1**, 373.

TABLE II.
Carbon and Hydrogen Analyses of the High Melting Compound.

| Compound | | | Calculated for: | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | H | $C_{27}H_{44}O$ | | $C_{28}H_{46}O$ | | $C_{30}H_{50}O$ | |
| | C | H | C | H | C | H | C | H |
| High melting | 84.35 | 11.73* | | | | | | |
| | 84.50 | 11.61* | | | | | | |
| | 83.43 | 11.38† | | | | | | |
| High melting recovered from digitonin ppt. | 84.02 | 11.55* | | | | | | |
| Avg | 84.06 | 11.57 | 84.31 | 11.53 | 84.35 | 11.63 | 84.44 | 11.81 |

* Analyses by Dr. A. J. Haagen Smit, California Institute of Technology.
† Analyses by Dr. H. L. Hunter, Eli Lilly and Company.

been added.[5] The results of the assay of the high melting compound (164-166°) are reported in Table I.

*b. Chemical properties.* The high melting compound gave a strong positive Liebermann-Burchard reaction. The compound could be precipitated with digitonin, and the original compound could be recovered from the digitonin precipitate by digestion with pyridine. The compound and its acetate showed an ultraviolet absorption spectrum (Fig. 1) with an inflection at 262-265 mu, and maxima at 272, 283, and 295.5 mu. The values of the extinction coefficient at the maxima were, for the high melting compound, respectively, 74.5, 77.1, and 45.5, and, for the acetate, respectively 35.6, 36.9, and 21.0. The carbon and hydrogen analyses of the high melting compound and of the product reisolated from the digitonin precipitation are given in Table II.

The acetate was prepared by refluxing 200 mg of the high melting compound for 30 minutes with 10 ml of acetic anhydride. The mixture was cooled and filtered. The crystals were washed, dried and recrystallized twice from 95% ethanol. The yield was 185 mg of colorless leaflets, melting at 141-143°, and having the following analysis:

| | | | |
|---|---|---|---|
| Found: | | C: 82.55 | H: 11.02 |
| | | 82.67 | 11.48 |
| | | 81.63 | 11.03 |
| | Avg | 82.35 | 11.18 |
| Calculated for: | $C_{30}H_{48}O_2$ | 81.76 | 10.98 |
| | $C_{32}H_{52}O_2$ | 81.94 | 11.18 |

The acetate was saponified according to Sandquist and Gorton[6] with the results shown in Table III. The high melting compound was hydrogenated with platinum oxide as catalyst and acetic acid as solvent with the results reported in Table IV.

TABLE III.
Equivalent Weight Determination of the High Melting Compound.

| Weight sample, mg | ml HCl, 0.100 N | Equivalent weight |
|---|---|---|
| 46.5 | 1.10 | 447.2 |
| 63.3 | 1.39 | 455.4 |
| 55.2 | 1.24 | 445.2 |
| Avg | | 449.3 |
| Calculated for: | $C_{29}H_{46}O_2$ | 426.6 |
| | $C_{30}H_{48}O_2$ | 440.69 |
| | $C_{32}H_{52}O_2$ | 468.74 |

TABLE IV.
Hydrogenation of High Melting Compound.

| Compound | Catalyst, mg | Sample, mg | $H_2$ taken up, ml | Time of hydr. | Moles of H per mole compound |
|---|---|---|---|---|---|
| Cholesterol high melting compound | 3.025 | 11.711 | 0.684 | 30 min. | 1.00 |
| | 3.011 | 10.988 | 1.190 | 25 min. | 1.93 |
| | 3.268 | 11.887 | 1.301 | 44 min. | 1.89 |
| | 2.878 | 14.872 | 1.629 | 10 hr | 1.93 |

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[5] van Wagtendonk, D. J., and Wulzen, R., *Arch.* **155**, 337.

[6] Sandquist, H., and Gorton, J., *Ber.*, 1930, **63**B, 1935.

The acetate was hydrogenated in the same manner. The product of the hydrogenation was recovered from the solution and recrystallized once from 95% ethanol. The compound melted at 130.5-132.0° and had the following composition:

| | | C | H |
|---|---|---|---|
| Found: | | 80.52 | 12.02 |
| | | 80.71 | 12.03 |
| | Avg | 80.62 | 12.02 |
| Calculated for: | $C_{29}H_{50}O_2$ | 80.87 | 11.70 |
| | $C_{30}H_{52}O_2$ | 81.05 | 11.80 |
| | $C_{32}H_{56}O_2$ | 81.29 | 11.94 |

*Summary.* A compound of steroid nature, having the probable formula of $C_{28}H_{46}O$, has been isolated from cane juice. This compound has therapeutic activity in alleviating an induced stiffness in guinea pigs. It has not been possible to further elucidate the structure of this compound, since not enough material was available at the time the project had to be discontinued, due to circumstances beyond control of the two senior authors. The isolations, however, tend to confirm the findings by Oleson *et al.* and Petering *et al.*, in that the antistiffness factor is steroid in nature.

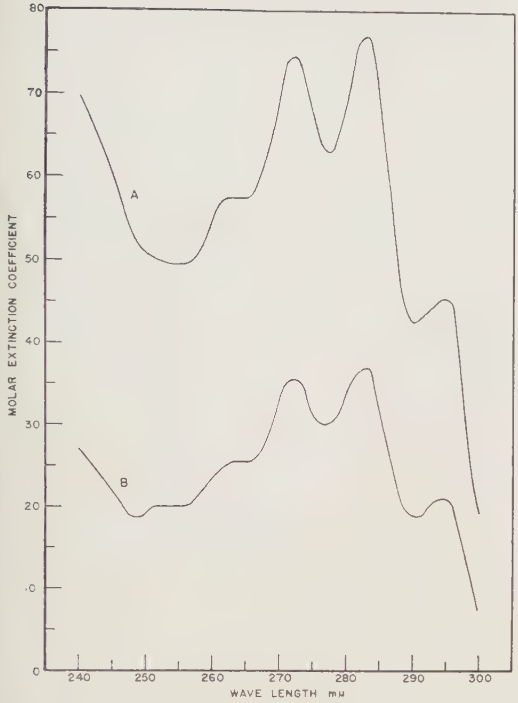

Fig. 1.

Ultraviolet adsorption of the high melting compound (curve A) and its acetate (curve B).

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## 17165. Electron Microscope Studies of the Vesicle and Spinal Fluids from a Case of Herpes Zoster.*

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(With the technical assistance of Ruth Peabody.)

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The pathogenesis of herpes zoster has not been resolved. Even though recent work has suggested that the virus may multiply in the skin, the part played by the central nervous system in this infection is not clear. Like the virus of varicella, with which it is either closely related or identical, the virus of herpes zoster cannot readily be passed to laboratory animals, making work difficult in this disease. However, elementary bodies of varicella and herpes zoster have been described as occurring in vesicular fluids when such fluids have been examined in the optical microscope.[1-3] These bodies have been characterized more fully in the electron microscope by Ruska,[4] by Rake

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* Aided by a grant from the Medical Fluid Research Fund of the Yale University School of Medicine.

† National Institutes of Health Postdoctorate Research Fellow.

Appreciation is expressed to Dr. John Craig for aid in obtaining specimens.

[1] Paschen, E., *Hyg. Rundschaw*, 1919, **29**, 269, 313.

[2] Aimes, C. R., *Lancet*, 1933, **224**, 1015.

[3] van Rooyen, C. E., and Illingsworth, R. S., *Brit. Med. J.*, 1944, **2**, 526.

[4] Ruska, H., *Scientia*, 1943, **37**, 16.

and his coworkers,[5,6] and by Farrant and O'Connor.[7] We have been able to confirm these findings in regard to finding elementary bodies in the vesicular fluids of varicella and of herpes zoster, and in addition have demonstrated particles of similar size in the cerebrospinal fluid of a patient with herpes zoster. The purpose of this paper is to present these findings and to compare the particles with those found in the vesicular fluid from a case of herpes simplex.

*Case report.* The patient studied was a 28-year-old post-graduate student who was admitted to the New Haven Hospital on December 14, 1948 with complaints of frontal headache of 10 days duration becoming more severe in the last 5 days, and of fever of 3 days duration. He had not noted chest pain or a skin rash. Previous hospital records disclosed that he had been under investigation for an unexplained splenomegaly and hyperglobulinemia since 1944. Physical examination, including a neurological evaluation, was not remarkable except for a group of early vesicles on an erythematous base which extended from the 9th and 10th thoracic vertebra posterially nearly to the mid-line anteriorally on the left side over the 6th and 7th ribs, and a spleen that was palpable 2 cm below the costal margin. There was no hyperaesthesia over the affected skin areas. Laboratory work on the day of admission revealed a normal white, red, and differential count. A lumbar puncture done on the following day yielded clear fluid with the following characteristics: leucocyte count 145 per cmm, all of which were mononuclear, protein 156 mg%, Wassermann negative and bacterial culture negative. Initial pressure was 170 mm of water, final pressure, 120. A second spinal puncture on the next day revealed an increase in cell count to 225, which were predominantly lymphocytes. The patient remained asymptomatic except for headache, but had daily fever up to 102°F for 4 days. Convalescence was uneventful.

*Materials and methods.* For the electron microscope preparations, vesicle fluid from the patient was obtained with a tuberculin syringe on the day following admission. It was collected in 0.5 ml of saline, and after a clearing centrifugation at 2000 r.p.m. for 20 minutes was spun at 18,000 r.p.m. for 30 minutes in a cooled rotor, 6 inches in diameter. The sediment was resuspended in 0.2 ml of distilled water. Two samples of cerebrospinal fluid of this patient in amounts of 2 and 8 ml were handled in a similar fashion as were the samples of vesicular fluid from cases of varicella. The specimen of herpes simplex fluid shown was obtained from a lesion on the upper lip of a patient with a respiratory infection. It was collected in 0.2 ml of saline and examined without centrifugation. Spinal fluid from 20 patients with various ailments (tuberculous meningitis, poliomyelitis, and noninfectious neurological disorders) were examined, 15 without centrifugation and 5 after centrifugation as above.

Small drops of the samples were placed on collodion covered 200-mesh stainless steel grids and allowed to dry. They were then shadowcast with chromium at an angle of 1 to 7, and examined in a RCA electron microscope, type EMU. The magnification was calibrated by photographing a silica replica of a diffraction grating (15,000 lines per inch) and by the

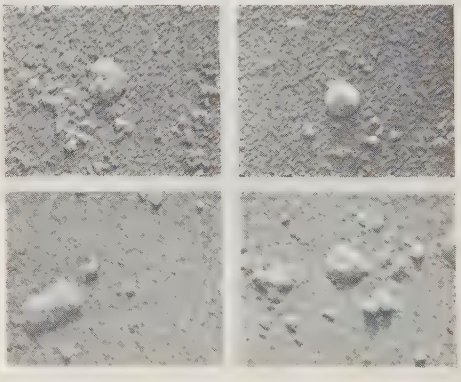

FIG. 1.
Electron micrographs of vesicle fluid from a patient with herpes zoster. × 20,700.

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[5] Nagler, F. P. O., and Rake, G., *J. Bact.*, 1948, **55**, 45.

[6] Rake, G., Blank, H., Coriell, L. L., Nagler, F. P. O., and Scott, T. F. M., *J. Bact.*, 1948, **56**, 293.

[7] Farrant, J. L., and O'Connor, J. L., *Nature*, 1949, **163**, 260.

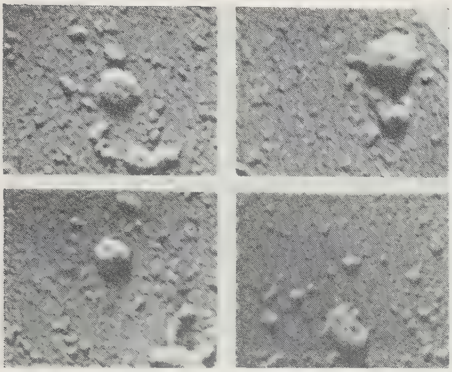

FIG. 2.
Electron micrographs of spinal fluid from a patient with herpes zoster. × 20,700.

use of latex balls of 259 mu diameter as described by Williams and Backus.[8]

*Results.* Fig. 1 illustrates typical bodies found in the vesicular fluid of the patient with herpes zoster and Fig. 2 those found in his spinal fluid. As seen from the frequency distribution of particles of varying diameters in Fig. 3, there is no significant difference between the average diameter of the elementary bodies found in either fluid, being 230 mμ for those in the vesicular fluid and 224 mμ for those in the spinal fluid.

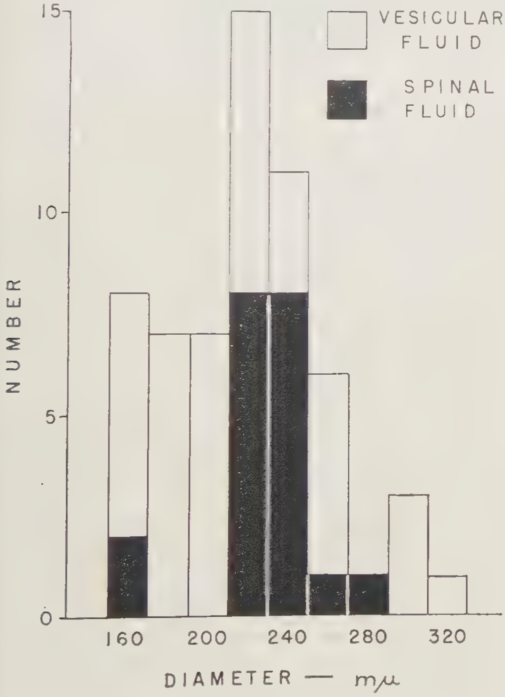

FIG. 3.
Frequency distribution of particles seen in the vesicular and spinal fluids of a case of herpes zoster.

No bodies of this sort were seen in any of the spinal fluids examined from patients with other diseases.

Fig. 4 illustrates elementary bodies obtained from vesicles of a patient with chicken pox, the average diameters of the particles being 244 mμ (range 200-280). The diameters of particles from 2 other patients with varicella averaged 250 mμ (range 190-320) and 225 mμ (range 180-250). The overall average of the particles from these 3 patients was 245 mμ.

Fig. 5 is a micrograph of elementary bodies from vesicle fluid of a patient with herpes simplex showing elementary bodies having an average diameter of 213 mμ (range 200-220).

It is difficult from any of our photographs to speak of brick-shaped elementary bodies such as discussed by Rake *et al.*[5] However some of the particles appear to have corners and in the measurements the average diameter of each particle was used. The preparations seem to be characterized by surface irregularities and particularly a central area of depression in the middle of which often appears a small elevated round structure. The latter was particularly marked in the bodies from herpes simplex (Fig. 5).

*Discussion.* These observations tend to confirm those recently presented by Rake and his colleagues[6] as well as those of Farrant and O'Connor[7] bearing on the similarities in elementary bodies observed in vesicular fluids from patients with herpes zoster and with varicella. Moreover, in addition to finding elementary bodies in the vesicular fluid of a patient with herpes zoster we have found particles of similar size in the spinal fluid of this patient. It is noteworthy that in the absence of clinical evidence of central nervous system involvement other than headache this patient showed a pleocytosis of 145 and 225 cells (chiefly lymphocytes) per cmm on 2 successive days during his disease. In a series

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[8] Williams, R. C., and Backus, R. C., *J. App. Phys.*, 1948, **19**, 119.

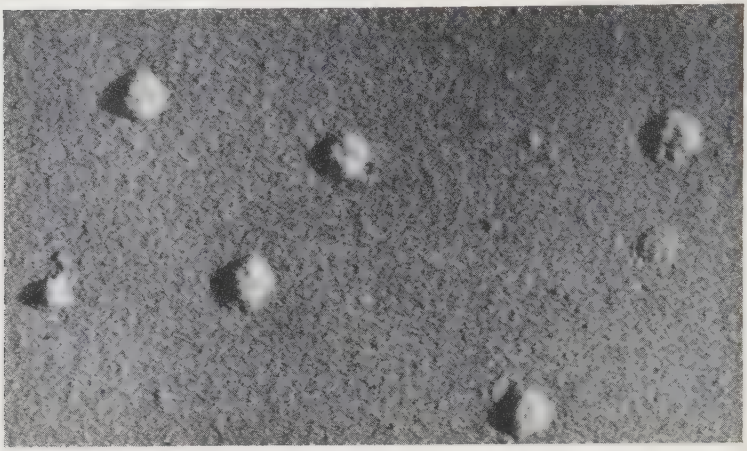

FIG. 4.
Electron micrographs of vesicle fluid from a patient with chicken pox. ×36,200.

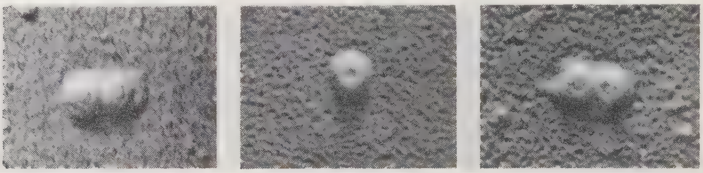

FIG. 5.
Electron micrographs of vesicle fluid from a patient with herpes simplex. × 27,500.

of 137 cases of herpes zoster reported by Gais and Abrahamson[9] lumbar punctures were done in 11: in 6 the count was normal while 5 showed a pleocytosis of 40 to 110 cells per cmm, chiefly lymphocytes.

Whereas herpes simplex appears to be unrelated to herpes zoster, similar appearing bodies have been detected in vesicular fluid here also. These bodies are 200-220 m$\mu$ in diameter which is considerably larger than that found by filtration studies of herpes virus contained in rabbit testicle and brain tissue by Elford *et al.*[10] However to our knowledge, filtration studies of the virus in vesicular fluid have not been done.

*Summary.* 1. Electron microscope studies of vesicular and spinal fluids from a patient with herpes zoster have demonstrated in both particles of similar size averaging 227 m$\mu$ in diameter.

2. These particles are compared to those in vesicular fluids from patients with herpes simplex and chicken pox. In herpes simplex particles averaging 213 m$\mu$ in diameter were found, and in chicken pox, particles of 245 m$\mu$.

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[9] Gais, E. S., and Abrahamson, R. H., *Am. J. Med. Sci.*, 1939, **197**, 817.

[10] Elford, W. J., Perdrau, J. R., and Smith, W., *J. Path. and Bact.*, 1933, **36**, 49.

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# 17166. Nature of the Plasma Factor Responsible for *In vitro* Lysis of Leucocytes by Tuberculoprotein.*

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*In vitro* tissue culture studies on the tuberculin reaction,[1] using washed buffy coats of sensitized animals, have stressed the role of cells rather than serum antibodies in the tuberculin type of hypersensitivity. Since the tuberculin reaction may be passively transferred to a normal host with white cells and not with the serum from sensitized animals,[2] and since white cell suspensions from tuberculous animals[3] and humans[4] are lysed by tuberculin within one hour, it would seem that the delayed type of hypersensitivity is in some way mediated via the white cells of the animal body.

In an earlier report[5] using the one hour observation technic on white cell suspensions it was noted that white cells of normal tuberculin-negative and tuberculous humans have an equal affinity for tuberculin antigen. Subsequent experiments[6] with the same technic, using thoroughly washed white cells of both normal tuberculin-negative humans and tuberculous patients, indicate that the one hour *in vitro* cytotoxic effect of tuberculin is dependent on the presence of some factor in tuberculous plasma. The present report extends these observations on the tuberculous plasma factor.

*Experimental.* Because normal white blood cells were shown to undergo the same lysis as do sensitized cells when incubated with tuberculin antigen and tuberculous plasma[6] and in order to eliminate any property of the tuberculous cell as a factor in cytolysis, thoroughly washed white cells from healthy tuberculin-negative human subjects were used in all experiments. Tuberculous plasma (or serum) was obtained from tuberculous patients hospitalized for acute tuberculosis. Normal white cell concentrates were diluted with this plasma in amounts sufficient to bring the final cell concentration to the range of 4,000-15,000 cells per cu mm. The tuberculous plasma was used in several ways:

a) freshly drawn;

b) after storage at 10°C for 7 days;

c) after dialysis for 2 days against isotonic saline;

d) after heating at 56°C for 15 minutes; and

e) as two fractions obtained as follows:

1. the globulin precipitated by dialysis of the plasma for 2 days against distilled water; and

2. the supernatant plasma freed of precipitate by centrifugation and redialized against isotonic saline. The supernatant plasma was added to one tube containing normal cells; the precipitate, after being suspended in 1 ml of isotonic saline, was added to a similar tube of normal cells.

In all cases where tuberculous plasma was subjected to heat or was used at any time subsequent to the day of collection from the patients, fresh normal guinea pig serum, containing approximately 400 units of active complement per ml, was added to the medium. To 0.4 ml of such white cell suspensions was

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* Work done under an U.S.P.H.S. Research Grant.

† Research Fellow, National Institute of Health.

[1] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

[2] Chase, M., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 134.

[3] Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 269.

[4] Fremont-Smith, P., and Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 502.

[5] Favour, C. B., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 369.

[6] Miller, J. M., Favour, C. B., Wilson, B. A., and Umbarger, M. A., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 738.

TABLE I.
*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Normal and Tuberculous Plasma Before and After Saline Dialysis at 10°C.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Normal cells | X | X | X | X | X | X | X | X |
| Tbc. Plasma (freshly drawn) | X | X | | | | | | |
| Normal Plasma (freshly drawn) | | | X | X | | | | |
| Tbc. Plasma (dialysis 48 hr against isotonic saline) | | | | | X | X | | |
| Normal Plasma (dialysis 48 hr against isotonic saline) | | | | | | | X | X |
| Tuberculin antigen | 0.1 | | 0.1 | | 0.1 | | 0.1 | |
| Saline | | 0.1 | | 0.1 | | 0.1 | | 0.1 |
| Total WBC | | | | | | | | |
| 5 min. | 8,770 | 9,420 | 8,530 | 9,450 | 7,380 | 8,290 | 9,270 | 11,260 |
| 60 min. | 6,510 | 9,390 | 8,500 | 9,360 | 5,330 | 8,320 | 9,210 | 11,080 |
| % Decrement | —25.8 | —0.3 | —0.5 | —0.8 | —27.6 | +0.2 | —0.7 | —1.5 |

added 0.1 cc of tuberculin antigen (prepared as described earlier[5]). Cell system controls with isotonic saline replacing the tuberculin antigen were studied in similar manner. In every instance, normal plasma from a tuberculin-negative donor was subjected to the same treatment as was tuberculous plasma (heat, dialysis, etc.) and added to normal white cell concentrates in the presence of "Old Tuberculin" and saline. Cytolysis was demonstrated by doing total white counts before and after a 60 minute period of incubation at 37°C. The accompanying protocols (Tables I, II, III) illustrate the method of experimentation. Essentially similar results have been obtained many times on different tuberculous patients.

*Results.* 1. Washed white cells from normal tuberculin-negative humans undergo significant cytolysis when suspended in the plasma of tuberculous patients in the presence of "Old Tuberculin" under conditions of these experiments (Table I).

2. The ability of tuberculous plasma to produce cell lysis is retained after dialyzing such plasma against isotonic saline for 48 hours at 10°C (Table I).

3. Heating tuberculous plasma to 56°C for 15 minutes destroys its ability to lyse white cells, even when fresh guinea pig complement is added to the system (Table II).

TABLE II.
*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Tuberculous Plasma Before and After Heating Plasma to 56°C for 15 Minutes.

| | | | | |
|---|---|---|---|---|
| Normal cells | X | X | X | X |
| Tbc. plasma | X | X | | |
| Tbc plasma* (heated 15 min. at 56°C) | | | X | X |
| Tuberculin antigen | 0.1 | | 0.1 | |
| Saline | | 0.1 | | 0.1 |
| Total WBC | | | | |
| 5 min. | 7,790 | 7,470 | 7,310 | 6,730 |
| 60 min. | 5,840 | 7,540 | 7,270 | 6,720 |
| % decrement | —24.8 | +0.8 | —0.8 | —0.3 |

* Fresh guinea pig complement added.

4. If tuberculous plasma is dialyzed at 10°C for 48 hours against distilled water, a white precipitate appears. The supernatant plasma, after redialysis against isotonic saline, has no effect on white cells in the presence of "Old Tuberculin." The precipitate, when resuspended in isotonic saline, is capable of causing white cell cytolysis (Table III).

TABLE III.
*In vitro* Effect of Tuberculin on Normal Leucocytes in Presence of Normal and Tuberculous Plasmas Before and After Dialysis Against Distilled Water.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal cells | X | X | X | X | X | X | X | X | X | X | X | X |
| Tbc. plasma* | X | X | | | | | | | | | | |
| Normal plasma* | | | X | X | | | | | | | | |
| Supernatant of tbc. plasma | | | | | X | X | | | | | | |
| Supernatant of normal plasma | | | | | | | X | X | | | | |
| Globulin precipitate tbc. plasma | | | | | | | | | X | X | | |
| Globulin precipitate normal plasma | | | | | | | | | | | X | X |
| Tuberculin antigen | 0.1 | | 0.1 | | 0.1 | | 0.1 | | 0.1 | | 0.1 | |
| Saline | | 0.1 | | 0.1 | | 0.1 | | 0.1 | | 0.1 | | 0.1 |
| Total WBC | | | | | | | | | | | | |
| 5 min. | 6,670 | 6,600 | 6,930 | 6,840 | 5,200 | 6,230 | 9,050 | 8,930 | 6,670 | 7,030 | 7,840 | 8,050 |
| 60 min. | 5,130 | 6,580 | 6,900 | 6,850 | 5,200 | 6,190 | 8,870 | 8,880 | 4,800 | 7,060 | 7,770 | 8,000 |
| % decrement | —23.0 | —0.2 | —0.5 | +0.2 | 0.0 | —0.7 | —1.8 | —0.4 | —28.0 | +0.5 | —1.1 | —0.6 |

* Both plasmas stored at 10°C for 10 days.

5. Tuberculous plasma, after storage at 10°C for 7 days, retains its capacity for lysing white cells in the presence of "Old Tuberculin" (Table III).

*Discussion.* An earlier report[6] stressed the essential role of tuberculous plasma in the one-hour *in vitro* cytolysis of human white cells. The foregoing results now indicate that the active component of such tuberculous plasma is a non-dialyzable, heat-labile fraction which remains stable after storage for 7 days at 10°C. The inactivation of such plasma by heating to 56°C for 15 minutes is apparently not due to the destruction of complement since the addition of fresh guinea pig complement did not restore the leucocyte lysing activity of the heated plasma. The precipitation of this active factor by dialyzing tuberculous plasma against distilled water seems to place this lysing component in the globulin fraction of the plasma proteins. The heat lability of this plasma substance distinguishes it from the usual precipitating and agglutinating antibodies described in tuberculous plasma. It is of particular interest that Chase[4] was able to destroy the ability of tuberculous cells to transfer sensitivity passively if such cells were heated to 48° for 15 minutes. In fact, work now in progress suggests that such tuberculous cells may be the source of this lytic promoting factor in plasma. It is not known whether the cytolytic process herein described represents an *in vitro* counterpart of the tuberculin type tissue reaction.

*Summary.* There is present in the plasma of tuberculous humans a heat-labile, non-dialyzable component which is responsible for the *in vitro* lysis of washed leucocytes by tuberculoprotein. Further properties of this lytic producing substance are its stability at 10°C for at least 7 days and its precipitation with the globulin fraction of the plasma proteins.

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## 17167. The Precipitating Effect of Methanol on Viruses.*

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Many technics have been applied to the problem of concentration of viruses. Those which utilize chemical and physical methods have been well reviewed by others.[1-3] Recently Cox *et al.*[4] reported on the application of the protein-precipitating property of methyl alcohol to the concentration of influenza B virus. By virtue of what seems to be a denaturating effect on somatic proteins and an innocuousness for virus protein, this technic appears to offer some promise of providing a method which might be useful with a wide range of viruses. It requires careful control over methanol concentration, temperature of reaction, and pH of the elution fluid.

The technic of virus precipitation with methanol is described in detail by Cox *et al.* and the procedure used in this study is essentially unchanged. The virus suspenion is maintained at 0°C to 5°C in an ice water bath. Chilled methanol (C.P.) is slowly mixed into the virus suspension and shortly thereafter a precipitate appears. The precipitation reaction is permitted to proceed for 3 hours. The precipitate is then sedimented in a chilled angle centrifuge at 4600 rpm for 30 minutes. The supernatant fluid is tested for virus activity and, since it is usually relatively free of virus, it is then discarded. The precipitate is resuspended to any volume desired in 0.2 M citrate or phosphate buffer, and the virus is permitted to elute at room temperature for one hour. The suspension is then centrifuged at 2500 rpm for 15 minutes at room temperature. The resulting supernatant fluid contains most of the virus.

Five viruses† were studied with this methanol precipitation technic, *viz:* 10% saline emulsion of Eastern equine encephalomyelitis virus (EEE) in mouse brain; 10% saline emulsion of human poliomyelitis virus (MEF) in mouse spinal cord; and influenza B, Newcastle disease, and mumps viruses in allantoic fluid. The EEE and MEF viruses were tested in decimal dilutions (0.03 ml per mouse) for infectivity by intracerebral inoculation into 10 g Swiss mice. Fifty percent lethal end points were determined according to the method described by Reed and Muench.[5] The 3 remaining viruses were tested for activity by the Hirst technic of chicken rbc agglutination.[6] In studying these five viruses some uniformity of pattern seems to have been manifested, particularly concerning the optimum concentration of methanol, and the optimum pH level at which virus elution occurs. All experiments re-

TABLE I.
Methanol Concentration and Elution pH for Optimal Yield of Virus.

| Virus | Optimum<br>% methanol | <br>Elution pH |
|---|---|---|
| EEE | 25 | 7 |
| MEF | 25 | 8 |
| Mumps | 30 | 8 |
| Influenza B | 25 | 9 |
| Newcastle disease | 30 | 7 |

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* Supported by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Anderson, T. F., *Cold Spring Harbor Symposia on Quantitative Biology*, 1946, **11**, 1.

[2] Pirie, N. W., *Annual Rev. of Biochem.*, 1946, **15**, 573.

[3] Gard, Sven, *Acta Med. Scand. Supp.*, 1943, **143**, 1.

[4] Cox, H. R., Van der Scheer, J., Aiston, S., and Bohuel, E., *J. Immunol.*, 1947, **56**, 148.

† Source of viruses:

MEF poliomyelitis—Dr. P. K. Olitsky, Rockefeller Institute, New York, N. Y.

Eastern equine encephalomyelitis—Local outbreak in equines during fall of 1947.

Mumps—Dr. J. F. Enders, Children's Hospital, Boston, Mass.

Influenza B—Dr. Joseph E. Smadel, Army Medical School, Washington, D.C.

Newcastle disease—Dr. L. T. Giltner, U.S.B.A.I., Pathological Division, Washington, D.C.

[5] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[6] Hirst, G. K., *Science*, 1941, **94**, 22.

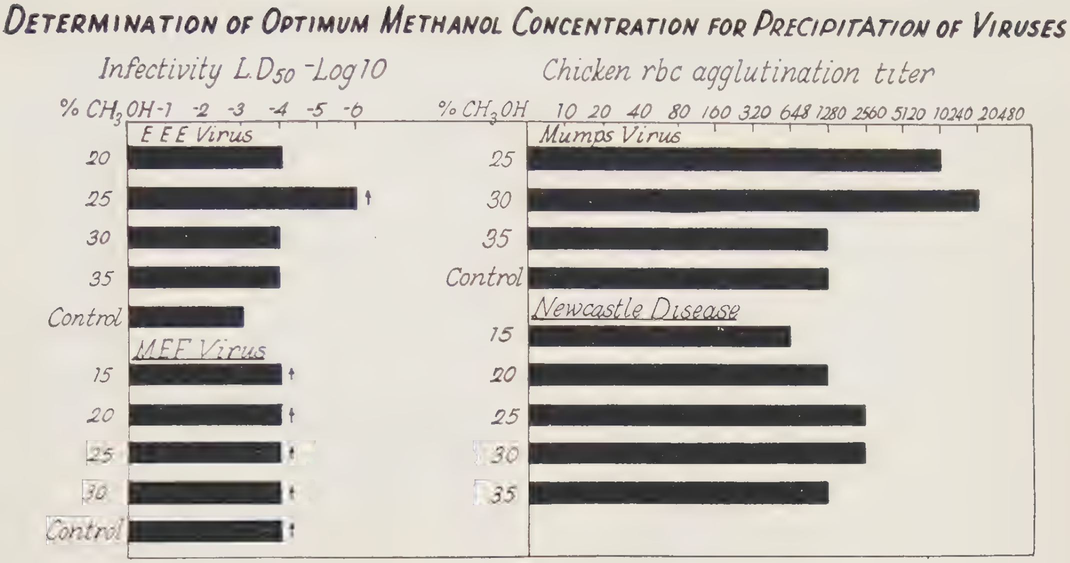

FIG. 1.

ported herein were repeated at least once, and the results were sufficiently in accord so as to warrant being published.

The optimum concentration of methanol was determined by adding 20, 25, 30, and 35% of methanol to aliquots of the virus suspension. By testing the resulting virus eluates in pH 7 buffer, the optimum methanol concentrations were arrived at and are listed in Table I: 25% methanol was optimal for EEE and MEF;‡ 30% for mumps virus and Newcastle disease virus. The concentration of methanol to be used for MEF virus was determined on the basis of complete extraction of the agent from the suspension by the precipitate and on this basis, 25% was found to be optimal. (Fig. 1).§ In accord with a previous report,[4] 25% methanol concentration was found to be optimal for influenza B virus.

Using the optimum concentration of methanol, as determined above for each virus, the pH at which each of the viruses would be eluted from the precipitate (or redissolved) most effectively was determined (Table I). It can be noted that the eluates were more potent as the pH of the eluting fluid was raised (Fig. 2). Each of the 5 viruses appeared to be more soluble in buffers at pH 7 or above than with buffers under pH 7. It was thought that some viruses might be inactivated at the lower pH levels, and so explain the lower titers of the eluates. However, when a precipitate containing EEE virus was eluted at pH 5 and then reeluted at pH 7, the virus was absent in the first elution but was recovered in the second step.

When virus suspensions were precipitated with methanol, the final eluate was found to contain less nitrogen than the original suspension. When the virus suspension was concentrated by reducing the relative volume of the final eluate, there was no proportional increment in content of nitrogen, while the virus content per ml was considerably increased. In fact, while there was considerable loss of nitrogen as a result of methanol precipitation, this loss did not appear to be at the expense of the virus. Table II illustrates some of the results of such microkjeldahl determinations.

*Discussion.* It can be noted that these methanol treated viruses have yielded eluates which are inordinately more potent than the

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‡ In a recent report Gollan indicates that 33% methanol was suitable for precipitating rodent MM poliomyelitis virus, PROC. SOC. EXP. BIOL. AND MED., May 1948, **67**, 3.

§ The arrows in Fig. 1 and III indicate that an end point was not determined.

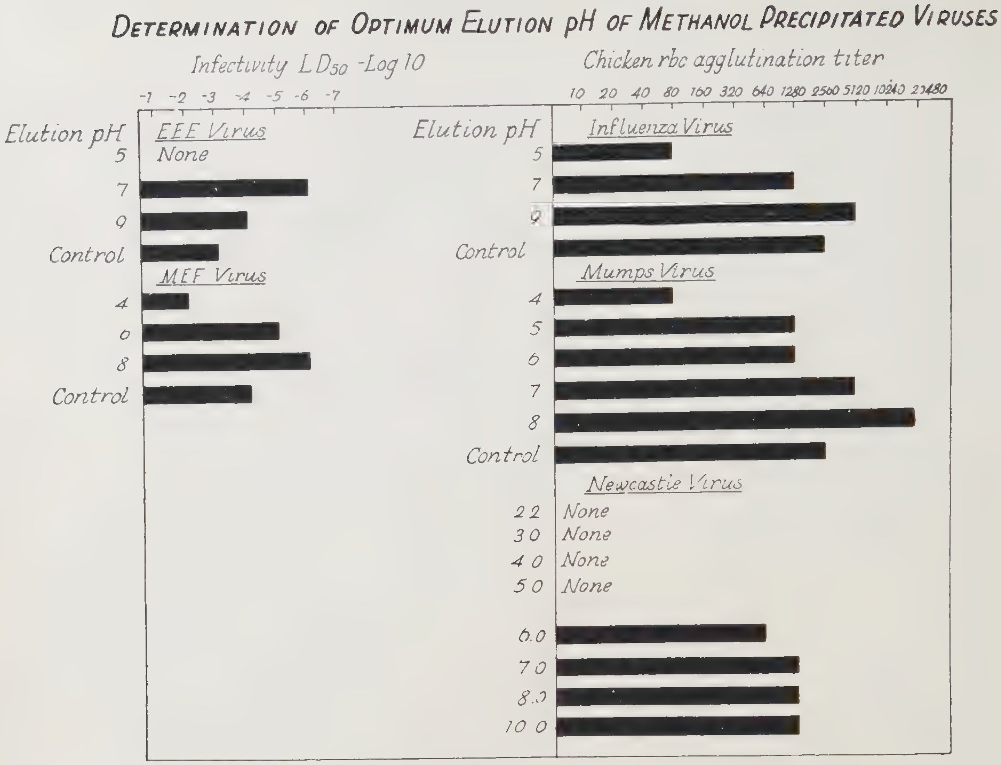

FIG. 2.

concentration factor would indicate. Our work has not yet offered any explanation for this phenomenon. The enhancement might be due to the disruption of infective aggregates or to the removal of some inhibiting substances as a result of this treatment with methanol. It may be that the viruses are manifesting an optimum pH activity point; however, Wenner[7] has reported that pH 4.6 is the optimum effective point for poliomyelitis. At this pH level, lower titratable endpoints were demonstrated in the eluates from methanol precipitated virus suspensions than were attained at the higher pH levels (Fig. 2). Horsfall[8] refers to autointerference agents which might provide an explanation for the enhancement of potency of the viruses following treatment with methanol. In all instances the nitrogen content of the virus suspension was reduced following methanol treatment, but not at the expense of the infective agent. This is in accord with the findings of Koprowski *et al.*[9] with methanol-precipitated viruses.

It may be that the viruses are less soluble at the lower pH levels because of the proximity to their isoelectric points; and in accordance with what has been demonstrated with enzyme proteins[10] and with animal serum proteins,[11] the solubility of the viruses increases as one moves away from this pH. As indicated above, while EEE virus was insoluble it was not destroyed after exposure

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[7] Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 104.

[8] Horsfall, Frank L., Jr., Viral and Rickettsial Infections of Man, J. B. Lippincott Co., Philadelphia, 1948.

[9] Koprowski, H., Black, J., and Cox, H. R., Proc. 4th Internat. Congress for Microbiol., July 1947.

[10] Northrop, J. H., Crystalline enzymes, Columbia University Press, 1939.

[11] Cohn, E. J., and Edsall, G., Proteins, Amino Acids and Peptides, Reinhold Publishing Corp., New York, 1943.

TABLE II.
Nitrogen Loss by Precipitation of Virus Suspension with Methanol.

| Virus CAF* | Mg N crude | Fold. conc. | Mg N after precipitation | % N reduction |
|---|---|---|---|---|
| Newcastle | .64 | 0 | 0.20 | 68.8 |
| ’’ | .62 | 0 | 0.35 | 43.8 |
| ’’ | .62 | 10 | 0.42 | 99.2 |
| Influenza B | .54 | 0 | 0.21 | 62.0 |
| ’’ A | .47 | 0 | 0.14 | 70.3 |
| ’’ A | .47 | 10 | 1.12 | 76.2 |
| Mumps | .56 | 100 | 2.91 | 94.9 |
| ’’ | .56 | 100 | 1.47 | 97.3 |
| ’’ | .56 | 100 | 0.35 | 99.3 |

* CAF — Chorio-allantoic fluid.

to pH 5 for one hour, and it was thereafter recovered with appropriate pH solutions. It has been reported that poliomyelitis virus can tolerate exposure to pH 1.6,[12] and on this basis it seems probable that the MEF virus was not destroyed when it failed to elute at the lower pH levels. Additional studies of the pH stability of MEF poliomyelitis virus indicate that while the virus was most stable at pH 6-7 it was able to tolerate pH 3 for 10 weeks.[13]

*Summary.* Five viruses (Eastern equine encephalomyelitis, MEF human poliomyelitis, mumps, influenza B, and Newcastle disease) were utilized in studying the precipitating effect on them of methanol. Optimal methanol concentrations and optimal pH for elution were determined for each virus and are tabulated (Table I). All of the viruses which were studied demonstrated a relationship between the pH of the elution fluid and solubility: as the pH was raised there was a corresponding increase in solubility of the virus. The methanol precipitation technic results in considerable loss of nitrogen from the virus suspension, but this loss does not appear to be at the expense of the virus.

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[12] Loring H., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 173.

[13] Pollard, M., and Connolly, J., Tex. Reports on Biol. and Med., Spring 1949.

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## 17168. Comparison of Newcastle Virus in Hamsters Exposed by Intracerebral Injection and Intranasal Instillation.

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Hamster-adapted Newcastle virus, California strain No. 11,914, has been continued through the 300th passage by serial intracerebral inoculation. Virus neutralization tests were conducted, using brain suspensions of the 245th and 300th intracerebral hamster passages with specific Newcastle virus immune and normal chicken sera, and with hamsters as test animals. Specific immune sera completely neutralized the hamster brain virus, whereas normal chicken serum had no effect. The virus of this 300th passage titred $10^{-4.77}$ intracerebrally in 4-week-old hamsters, in contrast to the virus of the 200th passage[1] which titred $10^{-4.25}$ in hamsters of the same age injected by the same route.

Newcastle virus infection in hamsters inoculated intranasally with 10% brain suspension of the 16th intracerebral hamster passage has been previously reported.[2] It was also reported that intranasal instillations

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[1] Reagan, R. L., Lillie, M. G., Hauser, J. E., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 293.

[2] Reagan, R. L., Lillie, M. G., Poelma, L. J., and Brueckner, A. L., *Am. J. Vet. Res.*, **8**, 427.

TABLE I.
Intranasal Passage of Hamster-Adapted Newcastle Virus.

| Passage No. | No. inoculated | No. showing symptoms | No. days after inoculation symptoms occurred |
|---|---|---|---|
| 1 | 6 | 5 | 3, 3, 4, 4, 5 |
| 2 | 5 | 5 | 2, 2, 3, 3, 4 |
| 3 | 8 | 6 | 4, 4, 4, 4, 5, 5 |
| 3a* | 5 | 2 | 3, 3 |
| 4 | 6 | 6 | 4, 4, 6, 6, 6, 6 |
| 4a | 6 | 2 | 4, 5 |
| 5 | 4 | 1 | 3 |
| 6 | 4 | 1 | 4 |
| 7 | 5 | 3 | 4, 4, 5 |
| 7a | 6 | 2 | 4, 4 |
| 8 | 4 | 2 | 3, 4 |
| 9 | 5 | 0 | |
| 9a | 8 | 8 | 3, 4, 4, 4, 4, 4, 4, 4 |

* "a"—Duplicate passages made from suspensions which were frozen and thawed.

TABLE II.
Titration in 4-Week-Old Hamsters of Hamster-Adapted Newcastle Virus.

| Virus | Dilutions | | | | | Titer[3] |
|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | |
| Hamster brain<br>300th passage | — | 4/4 | 4/4 | 3/4 | 2/4 | $10^{-4.77}$ |
| Hamster cord<br>300th passage | 4/4 | 4/4 | 0/4 | 0/4 | | $10^{-2.5}$ |
| Hamster brain<br>from 4th intranasal passage | 4/4 | 4/4 | 4/4 | 3/4 | 2/4 | $10^{-4.77}$ |
| Hamster brain<br>from 7th intranasal passage | 6/6 | 4/4 | 4/4 | 1/4 | | $10^{-3.66}$ |

Numerator of fraction denotes number of deaths.
Denominator of fraction denotes number of hamsters inoculated.

of lung or brain suspension from the affected animals failed to produce symptoms of central nervous system involvement in hamsters of the primary passage. Repeated trials with intranasal instillation of 10-20% infected brain suspension after the 16th intracerebral passage and up to the 245th intracerebral passage failed to produce typical symptoms of Newcastle virus infection, except in the first exposure. When a 20% brain suspension of the 245th intracerebral subculture was passed intranasally in young hamsters, symptoms involving the central nervous system, similar to those observed after intracerebral injection of the hamster-adapted Newcastle virus, were observed. These intranasal instillations were continued through 9 uninterrupted passages. Virus neutralization tests were conducted, using brain suspension of this 9th passage with specific Newcastle virus immune and normal chicken sera, with hamsters as test animals. Specific immune serum completely neutralized the hamster brain virus while normal chicken serum had no effect. Symptoms of central nervous system involvement appeared between the 2nd and 6th days following intranasal exposure. Table I gives the results of these 9 passages. Table II gives results of titrations in 4-week-old hamsters of the hamster-adapted Newcastle virus from various sources.

*Discussion.* Similar symptoms were shown by hamsters infected by the intracerebral route and by intranasal instillation, despite the fact that the incubation period was much shorter by the first method. Virus of the higher numbered intracerebral passages, and including the 300th, produced marked nervous reactions within 24 hours after intracerebral injection. This was true when dilution was carried as high as 1 to 1000. Hamsters exposed by intranasal instillation of 20% brain suspension in amounts as small as 0.06 cc became infected. In exposure by intranasal instillation the incubation period was regularly increased and ranged from 2 to 6 days. Brain suspension from hamsters infected intranasally in the 4th passage titred $10^{-4.77}$ by intracerebral injection, whereas the same form of material from the 7th passage titred $10^{-3.66}$ by the same route. These titrations were made with 0.03 cc of the various brain suspension dilutions. Spinal cord of hamsters of the 300th intracerebral passage, injected in 10% suspension intracerebrally, produced typical nervous symptoms. This suspension of virus titred $10^{-2.5}$, as shown in Table II.

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[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, **27**, 493.

*Summary.* Infected hamster brains from the 300th intracerebral passage and from the 4th intranasal passage titred $10^{-4.77}$, whereas infected brains from the 7th intranasal passage titred $10^{-3.66}$. Positive Newcastle chicken serum neutralized the virus from the 245th and 300th hamster passage, whereas normal chicken serum had no effect.

Nasal instillation trials were conducted from the 16th and 300th hamster passage but were not successful (except for the 1st passage) until after the 245th passage.

The incubation period of hamsters infected by intranasal instillation is longer than by intracerebral injection. Hamster-adapted Newcastle virus was carried through 9 passages intranasally, and brain material from this 9th nasal passage was neutralized by positive Newcastle chicken serum. Hamsters infected intranasally and intracerebrally showed symptoms of irritability followed by involuntary motor reactions and paralysis.

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## 17169. The Stability and the Stabilization of Testicular Hyaluronidase.

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Over a period of years, numerous preparations of hyaluronidase have been stored in these Laboratories as bulk powder at room temperature without any appreciable loss in activity. It has been observed, however, that decreases in potency occur when the enzyme is stored under other conditions. We have therefore conducted an investigation of the factors which influence the stability of testicular hyaluronidase. This involved the development of an accelerated aging test and the use of stabilizing agents. The stability of hyaluronidase in bulk, in aqueous solution and in sterile vials has been studied at 10°C, at room temperature and under conditions of accelerated aging. The influence of various bacteriostatic agents on the lability of the enzyme is reported.

*Methods.* The turbidimetric method of assay employed in this investigation has recently been described in detail.[1] This procedure permits the use of hyaluronates of varying degrees of purity as substrates and gives consistent results because of the stabilization of the protein indicator and the prompt termination of the reaction by heat. The activity of the enzyme is expressed in turbidity reducing units (T.R.U.) which are defined as that amount of enzyme which will hydrolyze hyaluronic acid until it gives a turbidity corresponding to half the amount of substrate employed. Using this method of assay the units have numerical values lower than those obtained by other procedures: *e.g.* 100 TRU

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[1] Tolksdorf, S., McCready, M. H., McCullagh, D. Roy, and Schwenk, E., *J. Lab. Clin. Med.*, 1949, **34**, 74.

TABLE I.
Loss in Activity of Bulk Hyaluronidase.

| Potency of sample TRU/mg | 10°C | | 25-30°C | | 60°C | |
|---|---|---|---|---|---|---|
| | Time in mo. | % loss | Time in mo. | % loss | Time in mo. | % loss |
| 25 | 12 | 0 | 12 | 0 | 1 | 40 |
| 150 | 12 | 0 | 12 | 0 | 1 | 20 |
| 500 | 2 | 0 | 3 | 58 | 1 | 62 |

by this method might be as high as 200 TRU by other methods.

The bulk hyaluronidase was prepared from bull testes by ammonium sulfate and lead acetate fractionation[2,3] and dried from the frozen state. Concentrated solutions of hyaluronidase in pyrogen-free distilled water were sterilized by Seitz filtration and tested for stability at various dilutions. Dilute, sterile filtrates of hyaluronidase were dried in vials from the frozen state. Early experiments indicated that hyaluronidase was less stable in vials than in bulk. Human plasma was added to the hyaluronidase and found to be a suitable stabilizer. Its use however was discontinued due to possible contamination with the virus of infectious hepatitis. It has been noted that certain labile substances such as protein hormones could be stabilized by the use of gelatine.[4] Gelatine which had been purified for intravenous use was therefore added to the vials before drying. This material after drying could be reconstituted only with difficulty. A polypeptide derived from gelatine* was then employed.

The shelf life of hyaluronidase both with and without stabilizer was determined upon storage at room temperature and at 10°C. Since some of the preparations proved to be very stable, an accelerated aging test was devised in order to expedite the study of the stabilization of hyaluronidase. The increased rate of inactivation was studied at various elevated temperatures up to 60°C. Although hyaluronidase in aqueous solution is destroyed almost immediately at 60°C,[1] hyaluronidase in dry form is inactivated at this temperature at a rapid but measurable rate. The stability of bulk powder as well as that of the enzyme in vials was therefore determined by incubation at 60°C for various periods of time.

Hyaluronidase in solution and dried in vials was also tested for stability in the presence of such bacteriostatic agents as zephiran (alkyl dimethyl benzyl ammonium chlorides), phenyl mercuric nitrate and tegosept (methyl and propyl esters of para hydroxy benzoic acid).

*Results. I. Stability of Bulk Hyaluronidase.* The stability of bulk hyaluronidase is illustrated in Table I. Material assaying 25 TRU and 150 TRU per mg respectively was stable for more than one year when stored at 10°C and at room temperature. Material of higher potency (500 TRU per mg) was stable for several months at 10°C. Stored at room temperature, the 500 TRU material lost 58% of its activity in 3 months. It should be mentioned however, that this study was carried out between June and September, 1948, when the average room temperature was close to 30°C. At 60°C the bulk material, regardless of potency, lost considerable activity over a period of one month.

II. *Stability of Aqueous Solutions of Hyaluronidase.* Aqueous solutions of hyaluronidase in 2% concentration possessed remarkable stability at 10°C. As may be seen from Table II, sterile filtrates of an enzyme preparation assaying 150 TRU per mg were stable for more than 3 months when kept in the refrigerator.

The stability of dilute enzyme solutions was studied at 10°C with 2 preparations assaying 150 and 500 TRU per mg respectively. The

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[2] Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

[3] Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

[4] Bockmühl, M., Lindner, F., and Schaumann, O., U. S. Patent No. 2,050,558, 1936.

* We are grateful to Dr. D. Tourtellotte of the Knox Gelatine Protein Products, Inc., Camden, N. J., who supplied the first experimental batches of intravenous gelatine and informed us of the availability of, and supplied us with, the polypeptide.

TABLE II.
Stability of Concentrated Aqueous Solutions of Hyaluronidase at 10°C.

| Experimental* | Original assay, TRU/ml | Time at 10°C, mo. | Final assay, TRU/ml |
|---|---|---|---|
| STE8-gen-20 | 2580 | 3 | 2730 |
| ” 22 | 2640 | 3 | 2760 |
| STE9-gen-1B | 2590 | 3 | 2700 |
| ” 6 | 2670 | 2.25 | 2900 |
| ” 9 | 3220 | 2.5 | 3260 |

* Enzyme employed assayed 150 TRU/mg. Concentration of sterile filtrates approximately 2%.

TABLE III.
Loss of Activity of a Dilute Aqueous Hyaluronidase Solution with and without Stabilizer.

| | Without stabilizer | | With polypeptide | |
|---|---|---|---|---|
| Enzyme STII-24* | Time | Loss, % | Time | Loss, % |
| 10° | 7 wks | 50 | 7 wks | 0 |
| Room temp. | 7 days | 50 | 18 days | 50 |

* Starting material 500 TRU/mg in 0.028% solution (140 TRU/ml).

500 TRU material at a concentration of 0.28 mg per ml lost 50% of its activity in 7 weeks whereas at the same temperature, in the presence of 2 mg of polypeptide per ml, there was no loss in activity over the same period of time (Table III). At room temperature, the half-life of the enzyme was 7 days without stabilizer as compared to a half-life of 18 days in the presence of polypeptide.

*III. Stability of Hyaluronidase in Vials.* The lability of hyaluronidase dried from the frozen state in vials in the absence of stabilizer was studied using material assaying 150 TRU per mg and 500 TRU per mg. The vials contained either 5 to 10 TRU, 100 TRU or approximately 150 TRU of the enzyme and were stored at 10°C, at room temperature and at 60°C. Regardless of the amount of hyaluronidase in the vials, the enzyme was stable at 10°C for more than 7 months. At room temperature, losses were frequently observed after 4 months while at 60°C, losses were observed within hours.

The addition of gelatine resulted in a very marked increase in the stability of the enzyme. For vials containing 5 TRU of hyaluronidase, the half-life was 2.5 hours at 60°C without gelatine whereas there was only a very slight loss in the potency of the same preparation in 12 days in the presence of 2 mg of this protein. Unfortunately the contents of the vials were not readily soluble in distilled water.

It was found that a polypeptide produced by the partial hydrolysis of gelatine* was quite soluble after being dried from the frozen state. The stabilizing effects of this polypeptide were the same over a range of 1 to 7.5 mg per vial. Therefore, it was decided to use 2 mg per vial regardless of the quantity and purity of the enzyme. In the presence of this stabilizer the hyaluronidase showed no loss of activity at 10°C and at room temperature for more than 6 months. Some studies made at 60°C are demonstrated in Table IV and are typical of our findings. The results given in the table are expressed in terms of half-life of the enzyme. It will be seen that the half-life of 500 TRU material under these conditions was increased 16 fold. The 150 TRU material had an average increase in half-life from 6.9 to 156 days, indicating a 23 fold increase in stability due to the polypeptide. The vials of batch number 8-gen-17 E contained only small quantities of the enzyme but were stabilized to the same extent as were the other batches. More highly purified hyaluronidase (FVIV-38 C) lost all activity during drying in the absence of stabilizer when the charge was 10 micrograms representing 5 TRU. No activity was lost in the presence of polypeptide. Since the enzyme without polypeptide was stable at room temperature in vials for a period of 4 months, it would seem possible that in the presence of polypeptide the stability at room temperature might be increased to a period of years.

TABLE IV.
Half-Life of Hyaluronidase in Vials at 60°C.

| Batch No. | Potency | Vials filled to contain | Half-life: Without stabilizer, days | Half-life: With stabilizer, days |
|---|---|---|---|---|
| FV IV-38 A | 500 TRU/mg | 150 TRU/vial | 3.5 | 56 |
| 8-gen-17 C | 150 ” ” | 165 ” ” | 4.6 | 163 |
| 8-gen-17 A | 150 ” ” | 110 ” ” | 14 | 114 |
| 8-gen-17 E | 150 ” ” | 9 ” ” | 2 | 190 |
| FV IV-38 C | 500 ” ” | 5 ” ” | * | 85 |

* All activity lost during drying.

TABLE V.
Stability of Hyaluronidase in Vials During Freeze-Drying.

| Batch No | Potency | Content of vials before drying, TRU | Content of vials after drying: Without stabilizer, TRU | Content of vials after drying: With stabilizer, TRU |
|---|---|---|---|---|
| FV IV-38 A | 500 TRU/mg | 150 | 120 | 160 |
| 8-gen-17 C | 150 ” ” | 165 | 154 | 163 |
| 8-gen-17 A | 150 ” ” | 110 | 98 | 114 |
| 8-gen-17 E | 150 ” ” | 9 | 4.7 | 11 |
| FV IV-38 C | 500 ” ” | 5 | no activity | 5.7 |

Observations were made to determine whether or not a decrease in potency occurred while drying the enzyme in vials. The influence of polypeptide on possible losses during drying was also investigated. Five such experiments are summarized in Table V. In each experiment the same amount of enzyme was placed in the vials with stabilizer as in the control vials. It will be noted that in the absence of stabilizer, losses occured which increased as the amount of enzyme decreased. In the presence of 2 mg of polypeptide, these losses were not observed. During the investigation of solutions of hyaluronidase, it had been noted that the polypeptide did not influence the initial assay values. Therefore it is believed that these experiments indicate that the polypeptide exerts a protective influence over the enzyme during drying.

*IV. The influence of Bacteriostatic Agents on the Stability of Hyaluronidase.* Tegosept, zephiran and phenylmercuric nitrate were employed in maintaining bacteriostasis in preparing various batches of hyaluronidase. These substances were separated from the enzyme by dialysis prior to drying the final product from the frozen state. Satisfactory preparations were obtained using zephiran and phenylmercuric nitrate. Some preparations in which tegosept was employed became inactive.

*Summary.* 1. Testicular hyaluronidase in bulk assaying 150 TRU per mg is stable at room temperature for more than one year.

2. Hyaluronidase in concentrated aqueous solution is stable for several months at 10°C. Dilute solutions of the enzyme are less stable.

3. Hyaluronidase dried from the frozen state in vials can be stabilized by the addition of polypeptide. Its half-life at 60°C is thereby extended 10 to 20 times.

4. Certain bacteriostatic agents can be used throughout the production of hyaluronidase without decreasing the activity of the enzyme.

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# 17170. Sympatholytic Effects of Diethylaminoethanol in Man.*

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A recent report by Rosenberg and his co-workers[1] indicates that diethylaminoethanol, an *in vivo* decomposition product of procaine, may revert ventricular tachycardia to normal rhythm, suppress ventricular premature contractions, and induce transient hypotension. Since the mechanism of these effects was not determined the present investigation was instituted to inquire into the hemodynamic properties of the drug in man.

*Methods and results.* Both hypertensive and normotensive subjects were given diethylaminoethanol in doses of 2 to 5 g either by slow intravenous drip in 0.5% solution at a rate of 0.02 g per minute or by rapid intravenous injection of a 10% solution at a rate of 1.0 g per minute. Sympathetic vasopressor and digital vasoconstrictor reflexes were determined before and at various intervals after the drug according to methods previously described.[2] Doses of 5 g of diethylaminoethanol injected intravenously at a rate of 1 g per minute were followed by a reduction of basal arterial pressure and marked postural hypotension. Heart rate either did not change significantly or increased moderately. Accompanying the hypotensive response there was an inhibition of sympathetic vasopressor responses (Valsalva and tilt-back overshoots), as well as a reduction in reflex vasoconstrictor responses to "noxious stimuli" in the digits. In addition, a sudden increase in digital volume and pulse volume was observed. These hypotensive and sympatholytic effects were transient, the arterial pressure and sympathetic reflexes returning to normal within ½ to 1 hour after the drug was administered. Slow rates of administration of the drug and low dosage were not followed by such cardiovascular or sympathetic reflex changes. No serious toxic effects were noted.

*Discussion.* The observed sympatholytic properties of diethylaminoethanol in man probably are the basis for its hypotensive action as well as its effect on ventricular tachycardias. In the latter respect the drug is similar to certain other sympatholytic agents such as dibenamine,[3] ergotamine,[4] and dihydroergokryptine.[5] This correlation between adrenergic inhibition and the prevention or cessation of attacks of ventricular tachycardia seems to be limited to drugs which suppress the sympathetic system only; since in dogs under cyclopropane anesthesia the drug tetraethylammonium, which inhibits transmission through all autonomic ganglia, intensifies rather than prevents epinephrine induced ventricular tachycardia.[6]

Rosenberg and his co-workers demonstrated that high blood levels of diethylaminoethanol are not maintained due to rapid diffusion of the drug out of the blood stream.[1] The results of the present study suggest that the sympa-

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[1] Rosenberg, B., Kayden, H. J., Lief, P. A., Marks, L. C., Steele, J. M., and Brodie, B. B., *J. Pharm. and Exp. Therap.*, 1949, **95**, 18.

[2] Freis, E. D., Stanton, J. R., Culbertson, J. W., Halperin, M. D., Litter, J., Burnett, C. H., and Wilkins, R. W., *J. Clin. Invest.*, 1949, **28**, 353.

[3] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, **7**, 397.

[4] Allen, C. R., Stutzman, J. W., and Meek, W. J., *Anesthesiology*, 1940, **1**, 158.

[5] Stanton, J. R., Ware, P., and Stutzman, J., unpublished data.

[6] Stutzman, J. W., Pettinga, F. L., Fruggiero, E. J., and Maison, G. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 686.

tholytic and hypotensive effects are dependent upon a high blood level of the drug since slow rates of infusion at low dosage did not result in sympathetic inhibition. Even with large doses rapidly injected adrenergic inhibition was transient and disappeared at a rate similar to the observed disappearance of high concentrations of diethylaminoethanol from the blood. It was not possible to determine from the present experiments whether the site of sympathetic inhibition was central or peripheral.

*Summary and conclusions.* Doses of diethylaminoethanol sufficient to produce high blood concentrations resulted in a temporary inhibition of sympathetic vasoconstrictor reflexes in man. It is suggested that the effects of the drug on arterial pressure and ventricular tachycardias are secondary to its sympatholytic action.

Since preparation of this paper, Clark and Helpern (*Fed. Proc.*, 1949, **8**, 282) have reported on the ganglionic blocking action of diethylaminoethanol in dogs and cats. Their observations on the sympatholytic effects of the drug in animals are in essential agreement with the results of the present investigation in man.

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## 17171. Action of 1-Methyl-4-(3-Hydroxyphenyl)-4-Piperidyl Ethyl Ketone on the Gastrointestinal Tract.*

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One of the most active analgesic compounds reported by Kleiderer, Rice *et al.*[1] was 1-methyl-4-(3-hydroxyphenyl)-4-piperidyl ethyl ketone which will be referred to in this report as WIN 1539. This compound has the same basic phenyl-piperidine nucleus as Demerol (brand of isonipecaine), but contains a metahydroxy group on the phenyl ring and has a propionyl group in place of the carbethoxy substituent of Demerol. The analgesic activity of WIN 1539 is 5-10 times greater than that of Demerol.[1-4] Because of its high analgesic activity and its close chemical relationship to Demerol, the actions of WIN 1539 on the gastrointestinal tract have been investigated.

*Results.* A. *Effect on excised intestinal segments.* WIN 1539 and Demerol were compared for their activity against barium chloride and acetylcholine induced spasms of the rabbit ileum and against histamine induced spasms of the guinea pig ileum by the procedure described by Miller, Becker and Tainter.[5] The results are given in Table I. These data indicate that the spasmolytic activity of WIN 1539 is considerably greater than Demerol against all three spasmogenic agents.

B. *Effect on intestine in situ.* Kymographic recordings were made of a segment of the intact ileum of anesthetized dogs by a modification of the Barbour method as described by Jackson.[6] Aqueous solutions of the drugs

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* This work is part of an investigation carried out under the supervision of Dr. M. H. Seevers and submitted in a dissertation to the Graduate School of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report No. 981, Office of the Publication Board, Department of Commerce, Washington, D.C.

[2] Scott, C. C., Kohlstaedt, K. G., and Chen, K. K., *Anesth. and Analges.*, 1947, **26**, 12.

[3] Lewis, J. R., Dissertation, University of Michigan, 1949.

[4] Cahen, R. L., Epstein, H. J., and Dramentz, C. S., *J. Pharmacol.*, 1948, **94**, 328.

[5] Miller, L. C., Becker, T. S., and Tainter, M. L., *J. Pharmacol.*, 1948, **92**, 260.

[6] Jackson, D. E., *Experimental Pharmacology and Materia Medica*, 1939, C. V. Mosby Co., St. Louis, Mo.

TABLE I.
Relative *in vitro* Spasmolytic Properties of WIN 1539 and Demerol.

| Compound | Barium chloride induced spasms | | Acetylcholine induced spasms | | Histamine induced spasms | |
|---|---|---|---|---|---|---|
| | Effective dilution* | Relative activity % papaverine | Effective dilution* | Relative activity % atropine $SO_4$ | Effective dilution* | Relative activity % papaverine |
| WIN 1539 | 1:1,000,000 | 630 | 1:3,200,000 | 2.5 | 1:1,000,000 | 475 |
| Demerol | 1:300,000 | 200 | 1:98,000 | 0.08 | 1:205,000 | 98 |

* Values are averages from intestinal strips of 6 animals.

were injected into the exposed femoral vein. A total of 6 dogs was used in these experiments. The most marked effect on the intestine following the administration of WIN 1539 was a decrease in tonus. There was no significant change in amplitude or frequency of the contractions. Doses of WIN 1539 as low as 0.01 mg/kg produced a definite decrease in tonus. These small doses had no effect on blood pressure, heart rate or respiration. Larger doses (1-2 mg/kg) did not cause stimulation of the intestine but only a relaxation of longer duration. The results obtained in a representative experiment are shown in Fig. 1.

C. *Roentgenographic study of the rate of movement of a barium meal through the gastrointestinal tract.* The method used was a modification of the one described by Gershon-Cohen and Shay.[7] Adult male albino rats were starved for approximately 24 hours. They were then given orally a 50% suspension of barium sulfate in 0.5% gum tragacanth, by intubation in a dose of 0.5 cc/100 g of body weight. The analgesic compounds were injected subcutaneously as aqueous solutions immediately preceding the barium meal. The doses used are indicated in Table II. These doses had been found to produce approximately the same degree of analgesia in rats. Roentgenograms were made at 1, 2, 3 and 4 hours following the administration of the barium meal. The results are summarized in Table II. The time for complete evacuation was not determined, but the data obtained indicate that there is a delay in the emptying time of the stomach due to the action of each of the analgesic drugs. Morphine caused the greatest and Demerol the least delay. The delay resulting from WIN 1539 was almost as great as from morphine. Morphine appears to act somewhat longer than WIN 1539 on the gastrointestinal tract which is also true of its analgesic effect. Karr[8] reported that morphine slowed the passage of a carbon suspension in the intestine of rats and that Demerol was much less active in this respect. This is undoubtedly related to

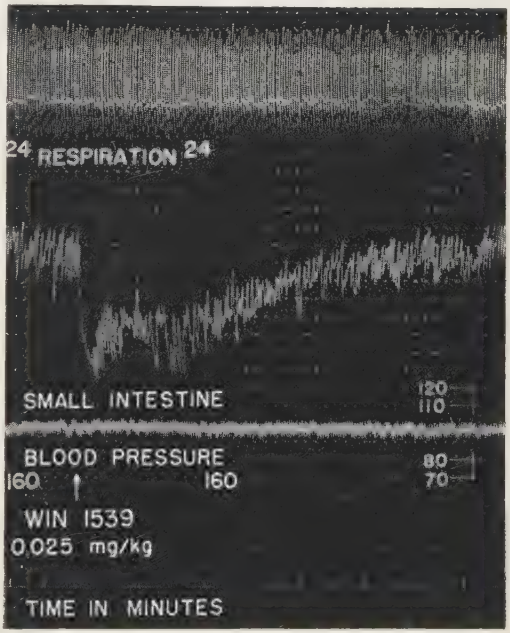

FIG. 1.
Action of WIN 1539 in an anesthetized dog. Upper figures are respiratory rates. Lower figures are heart rates. Scale at right represents mm Hg pressure.

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[7] Gershon-Cohen, J., and Shay, H., The Rat in Laboratory Investigation, 1942, p. 371, J. B. Lippincott Company, Philadelphia.

[8] Karr, N. W., *Fed. Proc.*, 1947, **6**, 343.

TABLE II.
Rate of Movement of a Barium Meal Through the Gastrointestinal Tract of Albino Rats.

| Drug | No. of animals | Approximate % barium meal remaining in stomach Hr after administration 1 | 2 | 3 | 4 | Location of remainder of barium meal 4 hr after administration |
|---|---|---|---|---|---|---|
| Untreated controls: | 7 | | | | | |
| Mean | | 15 | 9 | 7 | 4 | Cecum and feces. |
| Range | | 2-50* | 2-25 | 0-25 | 0-15 | |
| Morphine (10 mg/kg): | 5 | | | | | |
| Mean | | 100 | 96 | 86 | 49 | Jejunum and ileum. |
| Range | | — | 90-100 | 75-95 | 25-95 | |
| Demerol (30 mg/kg): | 9 | | | | | |
| Mean | | 58 | 46 | 34 | 26 | Lower ileum and cecum. |
| Range | | 20-100 | 10-95 | 2-95 | 2-90 | |
| WIN 1539 (3 mg/kg): | 10 | | | | | |
| Mean | | 89 | 70 | 46 | 44 | Jejunum, ileum and some in cecum of 4 animals. |
| Range | | 80-100 | 20-95 | 10-80 | 5-80 | |

* 2% indicates a visible trace—other values are estimates of percentage of total volume as determined from area and density of barium meal.

the emptying time of the stomach as shown by our results.

D. *Effect on stomach in situ.* Since the roentgenographic study showed that the analgesic drugs retarded the emptying of the stomach, the effect of these drugs on the motility and tone of the intact stomach was investigated. Jackson's method[6] of recording contractions of the dog's stomach was modified for use with rats. The rats were anesthetized with sodium pentobarbital and the apparatus attached so that recordings of the pyloric portion of the stomach were obtained. The drugs were injected into an exposed saphenous vein.

A total of 12 animals were used in these experiments. Fig. 2 is a record obtained in a representative experiment. It was observed that WIN 1539 in a dose of 0.1 mg/kg caused a marked decrease in the motility of the stomach. In contrast, 1.0 mg/kg of Demerol produced an increase in tone and in some experiments also an increase in frequency of contraction. In two experiments morphine caused a response similar to that obtained with WIN 1539 in that the peristaltic waves were abolished.

The marked decrease in the motility of the stomach caused by WIN 1539 or morphine could account for the delay in the emptying time which is an important factor in the constipating action of morphine. Gruber, Hart and Gruber[9] reported that Demerol produced a stimulation of the stomach and pylorus in anesthetized dogs. We obtained similar results in rats. In clinical use Demerol does not have a constipating effect. Even though morphine has an inhibitory action on the stomach of some species of animals (Krueger, *et al.*[10]), Veach[11] found that it is predominantly motor to the human stomach. Because of species differences in the digestive processes it cannot be stated that the actions obtained with WIN 1539 in these experiments would be the same for men.

*Summary.* WIN 1539, a new potent synthetic analgesic related chemically to Demerol, was found to have a stronger spasmolytic action than Demerol on the stimulated isolated intestinal strip. A decrease in tone of the intact small intestine of the dog was observed. WIN 1539 caused a greater delay than Demerol in the emptying time of the rat's stomach

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[9] Gruber, C. M., Hart, E. R., and Gruber, C. M., Jr., *J. Pharmacol.*, 1941, **73**, 319.

[10] Krueger, H., Eddy, N. B., and Sumwalt, M., The Pharmacology of the Opium Alkaloids, U. S. Public Health Reports, Supplement No. 165, Part 1.

[11] Veach, H. O., *J. Pharmacol.*, 1937, **61**, 230.

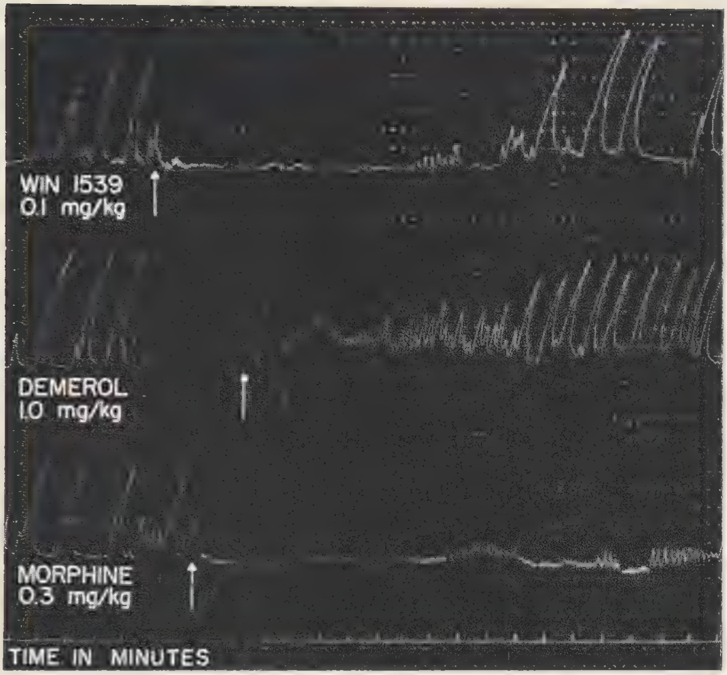

FIG. 2.
Kymographic recordings of intact stomach of anesthetized rat.

but morphine retarded emptying more than either of these drugs. Demerol caused a stimulation of the intact rat's stomach whereas WIN 1539 and morphine decreased the motility of this organ.

The author wishes to express his sincere appreciation to Dr. J. O. Hoppe, Mr. J. D. Frick, Misses B. L. Dertinger and Estelle Ananenko for their assistance in this work.

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## 17172. Some Relationships of Folic Acid to Structurally Similar Metabolites.[*]

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Jacobson and Good[1] observed that if folic acid (pteroylglutamic acid) is incubated with milk xanthopterin oxidase a material producing a greater response in cases of pernicious anemia than folic acid itself was obtained. At nearly the same time Kalckar and Klenow[2] demonstrated that xanthopterin is converted to leucopterin by milk xanthopterin oxidase and that this reaction is strongly inhibited by approximately equimolar amounts of folic acid. However, these workers observed that if folic acid is first incubated alone with the enzyme preparation the characteristic inhibition of xanthopterin oxidation is not observed.[3] They also showed later that this inhibitory effect is given to a much less extent by highly purified folic acid.[4]

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[*] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Jacobson, W., and Good, P. M., International Physiol. Congress, *Abstracts*, 1947, July.

[2] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 349.

Since the structure of the pterin nucleus is very similar to certain of the purines and also to the flavin prosthetic groups, it is possible that inhibition of enzymic reactions by folic acid is competitive in nature. Because of the same structural similarities the possibility also exists that folic acid can be metabolized to some extent by the same reactions. We have studied this problem in relation to both of these possibilities and have concluded that inhibition of purine metabolism *in vitro* by aldehyde-free folic acid does occur. Our results also indicate that folic acid itself may be metabolized to some extent by the same reactions.

*Experimental.* In order to observe the relationship between the *in vitro* metabolism of guanine, the purine most similar in structure to the pterin nucleus and folic acid, oxidation was followed using rat liver hemogenates prepared according to a method of Axelrod and Elvehjem.[5] Livers were obtained from adult, male albino rats maintained on stock ration for several months. The oxidation of guanine, folic acid and guanine plus folic acid was observed for 160 minutes using a conventional Warburg apparatus at 30°C. To each flask was added 1 ml of 16.6% liver homogenate. The levels of substrates employed were as follows: guanine, 0.2 ml 0.02 M; folic acid, 0.2 ml 0.02 M. In addition, 0.2 ml 1 x $10^{-4}$ M cytochrome-*c* and 0.2 ml water were added to each flask. When both guanine and folic acid were included in the same flask, the water was omitted. To absorb carbon dioxide, 0.2 ml 10% KOH was included in the center well of each Warburg vessel.

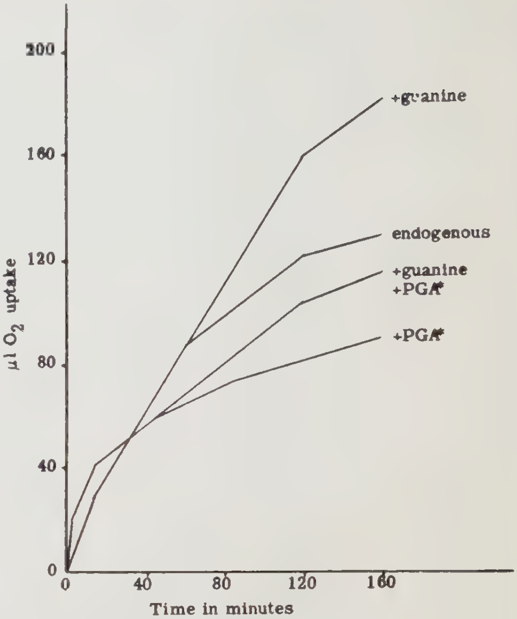

FIG. 1.

Oxygen uptake data showing the influence of folic acid upon guanine metabolism in rat liver homogenate.

* Aldehyde-free. See text.

Since oxidation of xanthopterin by milk xanthopterin oxidase has been shown to be strongly inhibited by samples of folic acid, a purified xanthine oxidase preparation diluted 5-fold with 0.039 M sodium potassium phosphate buffer (pH 7.5) was incubated with xanthine and xanthine plus folic acid (0.2 ml 0.02 M of each substrate). Oxygen uptakes were followed until oxidation of xanthine ceased.

Folic acid concentrations were made equivalent to concentrations of other substrates in respective experiments in order to observe relative oxidation of the substrates. Both commercial folic acid (folvite) and an aldehyde-free preparation of folic acid were employed separately to determine if aldehyde impurities are necessary for the inhibition of xanthine oxidase by folic acid. All substrates were made up in equivalent quantities of alkali.

*Results.* As shown by the curves in Fig. 1, folic acid strongly inhibits endogenous respiration in rat liver *in vitro*. Also metabolism of guanine (presumably via guanase to xanthine and subsequent oxidation of the xanthine) is decreased about 50%. In the experiment shown aldehyde-free folic acid was employed although almost exactly similar results were obtained when commercial folic acid was employed. Because of the structural similarities between guanine and folic acid it appeared possible that the pterin nucleus could be metabolized similarly to guanine, *i.e.*, a hydrolysis and subsequent oxidation. However, such an effect as this involving oxidation would be obscured by the depression of endogenous respiration by folic acid. It is interesting to note that in the early portion of the curve for flasks containing folic acid there is a brief stimulation of oxygen uptake.

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[3] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, 1948, **172**, 351.

[4] Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

[5] Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, **140**, 725.

The similarity of the pterin nucleus to the flavin prosthetic groups and purines may possibly explain the depression of endogenous respiration and oxidation of the xanthine arising from guanine. It is also possible that guanase itself is inhibited competitively by folic acid.

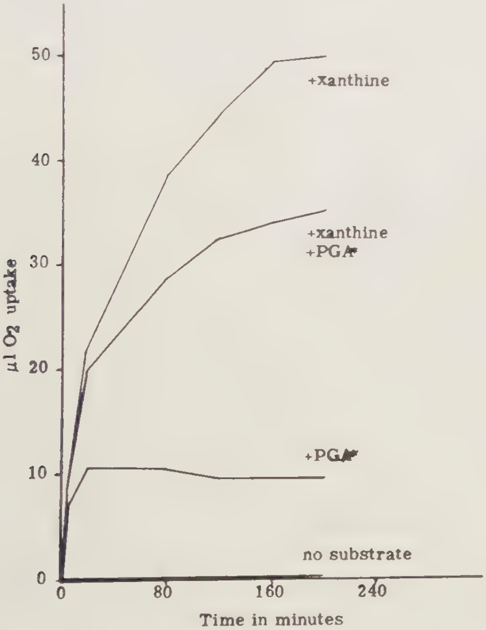

FIG. 2.

Oxygen uptake data showing the interrelationships of xanthine and folic acid in a purified xanthine oxidase preparation.

* Aldehyde-free. See text.

In Fig. 2 a typical experiment is presented in which a purified xanthine oxidase preparation was employed. Here oxidation of xanthine proceeds easily if it is present alone with the enzyme. However, this oxidation is strongly inhibited by folic acid. Some oxidation of folic acid appears to occur when it is included in the system alone with the enzyme. This oxidation, however, occurs very quickly and approaches a maximum in about one-half hour. This oxygen uptake might conceivably have arisen from oxidation of aldehydic impurities in the folic acid. However, in this experiment aldehyde-free folic acid was employed. Therefore, this small amount of oxidation appears to arise from oxidation of the folic acid itself, although the reaction is soon inhibited by the product or products formed. Whether the product of the reaction between folic acid and xanthine oxidase is identical with the "activated" folic acid of Jacobson is open to question at this time.

*Summary.* Folic acid whether commercial or aldehyde-free inhibits guanine metabolism and endogenous respiration in rat liver homogenates. Oxidation of folic acid itself in such systems is not clearly evident.

Oxidation of xanthine by a purified preparation of xanthine oxidase is strongly inhibited both by commercial and aldehyde-free folic acid. In a system containing only folic acid and the enzyme some oxidation of the folic acid appears to occur although this reaction is transient.

We are indebted to Dr. D. E. Green of the Institute for Enzyme Research, University of Wisconsin, Madison, for the xanthine oxidase preparation and aldehyde-free folic acid and to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for the folvite. The aldehyde-free folic acid was a gift from the Lederle Laboratories to Dr. D. E. Green.

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## 17173. Inhibition of the Antimalarial Activity of Chlorguanide by Pteroylglutamic Acid.

JOSEPH GREENBERG. (Introduced by N. B. Eddy.)

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Chlorguanide (1-(p-chlorophenyl)-5-isopropylbiguanide) is a widely used antimalarial compound, the activity of which was first described by Curd, Davey and Rose.[1] It has been shown to have several interesting relationships with sulfadiazine. When the two drugs are given together there is definite potentiation of effect against *Plasmodium gallinaceum* in the chick.[2] Some chlorguanide-resistant strains are hypersensitive to sulfadiazine,[3] while others are resistant to sulfadiazine.[4] P-aminobenzoic acid will antagonize the antimalarial activity of sulfadiazine[5,6] but not that of chlorguanide.[4] The mode of action of these two drugs is thus not identical but is probably very closely related. It is known that pteroylglutamic acid (PGA) can inhibit the activity of sulfonamides.[7] Experiments were, therefore, undertaken to see if PGA would interfere with the activity of chlorguanide.

*Materials and Methods.* The tests were conducted according to a procedure previously described.[8] New Hampshire Red Chicks, maintained on a commercial starting mash (Purina Startena), were used throughout. The birds were one week old at the start of an experiment and weighed 42 to 45 g. Treatment was administered twice daily for 4 days beginning 4 to 5 hours before intravenous inoculation with 16 x $10^6$ chick erythrocytes parasitized by *P. gallinaceum*. Parasite counts (parasitized erythrocytes per $10^4$ erythrocytes) were made on the morning of the 4th day after inoculation of parasites (day of inoculation is day O). A dosage level of drug which causes a 75% reduction in parasitemia in the treated chicks as compared with untreated controls is considered the minimum effective dose. Chlorguanide hydrochloride was administered orally in aqueous solution by catheter and pteroylglutamic acid was administered at the same time and by the same route, in gelatin capsules.

*Experimental.* The minimum effective dose of chlorguanide has been found to be about 0.002 mg/g twice daily for 4 days. Pteroylglutamic acid alone has no significant effect on parasitemia. When the 2 compounds were administered together (Table I), pteroylglutamic acid at 0.002 mg/g did not inhibit the antimalarial activity of chlorguanide. However, PGA at 0.02 mg/g significantly but not completely inhibited the effect of chlorguanide; no further inhibition was produced by 0.2 mg/g. Larger doses of chlorguanide were partially inhibited by PGA.

Pteroylglutamic acid at 0.2 mg/g body weight completely inhibited the minimal effective dose of sulfadiazine, but it had no effect on the antimalarial activity of quinine, chloroquine or pamaquine (Table II).

*Discussion.* The fact that pteroylglutamic acid can inhibit the antimalarial activity of chlorguanide is not proof that chlorguanide is a direct antagonist of PGA. It is possible that the parasite may be able to use PGA to synthesize another metabolite with which

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[1] Curd, F. H. S., Davey, D. G., and Rose, F. L., *Ann. Trop. Med.*, 1945, **39**, 208.

[2] Greenberg, J., Boyd, B. L., and Josephson, E. S., *J. Pharm. Exp. Therap.*, 1948, **94**, 60.

[3] Greenberg, J., *J. Nat. Malaria Soc.*, 1949, **8**, 80.

[4] Bishop, A., and McConnachie, E. W., *Nature*, 1948, **162**, 541.

[5] Maier, J., and Riley, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 152.

[6] Marshall, E. K., Litchfield, J. T., Jr., and White, H. J., *J. Pharm.*, 1942, **75**, 89.

[7] Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1946, **164**, 485.

[8] Coatney, G. R., and Sebrell, W. H., In F. Y. Wiselogle's "A Survey of Antimalarial Drugs, 1941-1945." J. W. Edwards, Ann Arbor, Mich., 1946.

TABLE I.
Inhibition by Pteroylglutamic Acid of the Antimalarial Activity of Chlorguanide Against *Plasmodium gallinaceum* in the Chick.

| No. chicks | Dose of Chlorguanide HCl (mg/g b.i.d. for 4 days) | Dose of Pteroylglutamic acid (mg/g b.i.d. 4 days) | 4th day parasitemia (parasitized erythrocytes per $10^4$ erythrocytes) |
|---|---|---|---|
| Exp. 1 | | | |
| 10 | — | — | 8070 ± 175 |
| 5 | .002 | — | 1674 ± 364 |
| 5 | .002 | .002 | 990 ± 404 |
| 5 | .002 | .02 | 3540 ± 558 |
| 5 | .002 | .2 | 3425 ± 748 |
| Exp. 2 | | | |
| 10 | — | — | 6840 ± 319 |
| 5 | .002 | — | 44 ± 25.1 |
| 5 | .004 | — | 44 ± 36.0 |
| 5 | .008 | — | 6 ± 1.9 |
| 5 | .002 | .2 | 5140 ± 276 |
| 5 | .004 | .2 | 1858 ± 937 |
| 5 | .008 | .2 | 278 ± 219 |
| Exp. 3 | | | |
| 10 | — | — | 6790 ± 213 |
| 5 | .002 | — | 628 ± 197 |
| 5 | .002 | .02 | 4520 ± 1010 |
| 5 | .002 | .2 | 3790 ± 263 |

TABLE II.
Effect of Pteroylglutamic Acid on the Antimalarial Activity of Quinine, Pamaquine, Chloroquine and Sulfadiazine. (Five chicks were used in each group).

| | Dosage mg/g twice daily for 4 days | Dosage of pteroylglutamic acid mg/g twice daily for 4 days | 4th day parasitemia (parasitized erythrocytes/ $10^4$ erythrocytes) |
|---|---|---|---|
| Untreated | — | — | 7244 |
| Quinine hydrochloride | .01 | — | 6060 |
| | .02 | — | 942 |
| | .02 | .2 | 796 |
| Chloroquine diphosphate | .001 | — | 4196 |
| | .002 | — | 30 |
| | .002 | .2 | 8 |
| Pamaquine citrate | .0005 | — | 4762 |
| | .001 | — | 208 |
| | .001 | .2 | 108 |
| Sulfadiazine | .015 | — | 4120 |
| | .03 | — | 2170 |
| | .03 | .2 | 7280 |

chlorguanide directly competes. The fact that it requires about 5 molecules of PGA to inhibit significantly the effect of one molecule of chlorguanide, and that not even 50 molecules of PGA can completely inhibit the effect of one molecule of chlorguanide might indicate that chlorguanide is doing more than competing with PGA. However, the hypothesis that chlorguanide is a PGA antagonist is an appealing one in view of the relationship between chlorguanide and sulfadiazine, and between the latter and pteroylglutamic acid. In this connection we have found (unpublished data) that 2, 4-diamino-6, 7-diphenylpterin which is believed to stop bacterial growth by preventing the use of

PGA,[9] behaves similarly to chlorguanide in *P. gallinaceum* infections. It potentiates sulfadiazine to about the same degree as chlorguanide and its activity is partially inhibited by the same amounts of PGA as are required to inhibit chlorguanide. If chlorguanide should prove to be a PGA antagonist, this fact would be highly significant in the development of chemotherapeutic agents for malaria and other diseases as well as for the study of the metabolism of the malaria parasite.

*Summary.* The antimalarial activity of chlorguanide against *Plasmodium gallinaceum* in the chick could be significantly but not completely inhibited by pteroylglutamic acid *in vivo.* Pteroylglutamic acid had no significant effect on the antimalarial activity of quinine, chloroquine or pamaquine, but completely inhibited the effect of sulfadiazine.

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[9] Daniel, L. J., and Norris, L. C., *J. Biol. Chem.*, 1947, **170**, 747.

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## 17174. Pancreatic Islet Hyperplasia in Rats Force Fed High Carbohydrate Diets.

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Although both the cat and the dog may develop islet degeneration and irreversible diabetes after prolonged periods of hyperglycemia,[1-6] permanent diabetes has not been produced in the rat by methods short of partial pancreatectomy or alloxan treatment. After noting the ease with which Ingle produced chronic glycosuria in the rat by force feeding a high carbohydrate diet,[7] it occurred to us that it might be of interest to perform a similar experiment and examine the islets of Langerhans.

*Plan of experiments.* Two groups of 6 male albino rats of Sprague-Dawley strain were subjected to force feeding of gradually increasing quantities of high carbohydrate rations until glycosuria was sustained. Weights and urinary glucose excretion were determined at frequent intervals. Rats which died during the experiment as well those which survived for the entire experimental period of 56 days were examined grossly and histologically, especial attention being given the appearance of the pancreatic islets.

*Rations, method of feeding and urine glucose determinations.* A liquid ration containing about 50% carbohydrate was force fed to each group. The ration fed Group I (Table I) was slightly modified from that described by Ingle.[7] The ration fed Group II was similar except that the carbohydrate was all glucose. The latter diet was employed with the thought that intestinal absorption might be faster and more complete, thus making it possible to obtain higher levels of blood glucose. The rations were force fed 2 times per day at about 8 a.m. and 5 p.m. by means of the technic described by Shay and Gruenstein.[8] Starting with 6 ml per rat the daily portions administered were increased by 4 ml per day until 26 ml were being given. This quantity was maintained for a week to accustom the rats to the method of feeding and to avoid "food-shock".[7] Following this the quantities of ration were gradually in-

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[1] Homans, J., *J. Med Res.*, 1914, **30**, 49.

[2] Homans, J., *J. Med. Res.*, 1915, **33**, 1.

[3] Allen, F. M., *J. Metab. Res.*, 1922, **1**, 75.

[4] Copp, E. F. F., and Barclay, A. J., *J. Metab. Res.*, 1923, **4**, 445.

[5] Dohan, F. C., and Lukens, F. D. W., *Science*, 1947, **105**, 183.

[6] Dohan, F. C., and Lukens, F. D. W., *Endocrinol.*, 1948, **42**, 244.

[7] Ingle, D. J., *Endocrinol.*, 1946, **39**, 43.

[8] Shay, H., and Gruenstein, M., *J. Lab. and Clin. Med.*, 1946, **312**, 1384.

TABLE I.
Composition of Rations for Force Feeding.

| Constituents | Units | Ration I (Group I) | Ration II (Group II) |
|---|---|---|---|
| Egg albumin | g | 8 | 8 |
| Brewers yeast | ” | 5 | 5 |
| Corn starch | ” | 25 | — |
| Dextrin | ” | 12.5 | — |
| Glucose | ” | 12.5 | 50 |
| Ruffex | ” | 6 | 6 |
| Salt mixture | ” | 2 | 2 |
| Wheat germ oil | ” | 5 | 5 |
| Corn oil | ” | 10 | 10 |
| Oleum percomorphum | | | |
| Vitamin A | U.S.P. units | 200 | 200 |
| ” D | ” ” | 29 | 29 |
| ” K | mg | 5 | 5 |

Water to make total of 100 ml added to each ration.

creased as tolerated until 68 ml were being given daily. Each group of rats was housed in wire bottomed cages and allowed water *ad libitum*. Beginning on day 7 and on alternate days thereafter the rats of each group were placed in individual metabolism cages and their urine was collected under toluol for a 24-hour period for qualitative and quantitative glucose determinations. Qualitative determinations were performed with 0.5 ml of urine and 5 ml of Benedict's solution which were mixed and heated in a boiling water bath for 5 minutes. Quantitative urine glucose determinations were performed using Benedict's method when the qualitative test showed 2+ or more.

*Histologic preparations.* All tissues were fixed in Bouin's solution and imbedded in paraffin. The sections of pancreas were prepared in Dr. Gomori's laboratory and stained with the chrome hematoxylin and phloxine stain.[9] Hematoxylin and eosin stained sections of liver, stomach, kidney, thymus, and adrenal were examined from all animals and lung, skeletal muscle, mediastinum and testis were studied from selected animals.

*Experimental results.* The quantity of diet administered daily, the average body weights and the urinary glucose values for Group I are summarized in Fig. 1. It is apparent that increasing the diet intake from 26 ml per day to 68 ml per day resulted in a steady increase in body weight amounting to an average weight gain of almost 200 g for the 8 week period. Urinary glucose became apparent on the 15th day and was excreted by the majority of the animals of this group in quantities varying from 0 to 1.48 g per day for the remainder of the experiment. The animals of this group appeared in moderately good health and only one rat succumbed before the termination of the experiment, due to suffocation from aspirated diet.

The response of the rats of Group II to the ration containing only glucose as the carbohydrate source differed from those of group I, which received the mixed carbohydrate ration in the following respects: the animals tolerated this ration much more poorly and developed signs of food-shock on several occasions, necessitating the interruption of the steady increase of daily rations. Three of the 6 rats succumbed early in the experiment, 2 from food-shock and 1 from false passage of the stomach tube. Two of the remaining 3 rats died before the end of the experiment, 1 of food-shock and 1 of aspiration of diet. The 2 rats which survived beyond the 35th day of the experiment consistently showed more urinary glucose than the rats of Group I. Acetone was noted on 3 occasions in the urine of these rats.

At necropsy the only consistently noted gross change was marked obesity in rats which had been force fed either ration for 30 or more days.

The significant histologic findings in these

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[9] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

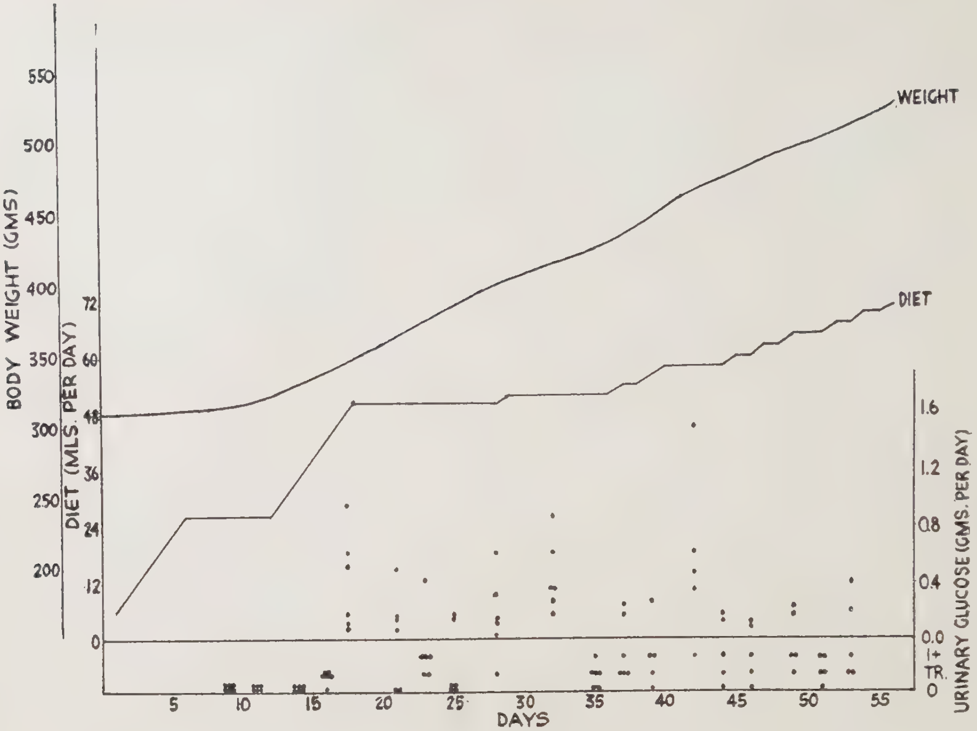

FIG. 1.
Weights, dietary intakes and urinary glucose excretion of rats force fed Diet I.

rats are summarized in Table II. The most striking changes were apparent in the islets of Langerhans. All animals which had received either ration for 32 days or longer showed marked hyperplasia of these structures, accompanied by a consistent decrease in granulation of the beta cells (Fig. 2 and 3). These changes were well developed in the rats which succumbed at 32 and 38 days and tended to increase as the rations were fed for longer intervals. In most instances the average islet diameters were 2 to 4 times greater than those of control animals. There was no evidence that the rats of Group II showed more marked

TABLE II.
Histological Findings in Rats Force Fed High Carbohydrate Diets.

| Group No. | Rat No. | Time force fed, days | Pancreas: Islet hyperplasia | Pancreas: Degranulation of beta cells | Adrenal cortical hyperplasia | Thymic atrophy | Liver: Glycogen | Liver: Fat |
|---|---|---|---|---|---|---|---|---|
| I | 1 | 55 | +++ | +++ | + | 0 | +++ | + |
| | 2 | 55 | ++++ | +++ | ++ | 0 | ++++ | 0 |
| | 3 | 55 | ++++ | +++ | + | + | ++++ | 0 |
| | 4 | 55 | +++ | +++ | ++++ | 0 | +++ | + |
| | 5 | 38 | +++ | +++ | ++ | 0 | + | 0 |
| | 6 | 55 | ++++ | +++ | 0 | + | +++ | ++ |
| II | 1 | 55 | +++ | +++ | ++ | 0 | ++++ | 0 |
| | 2 | 14 | + | ++ | 0 | 0 | + | 0 |
| | 3 | 10 | 0 | + | 0 | 0 | ++ | 0 |
| | 4 | 14 | + | ++ | +++ | 0 | + | + |
| | 5 | 32 | ++ | +++ | 0 | + | + | 0 |
| | 6 | 51 | +++ | +++ | 0 | 0 | ++++ | 0 |

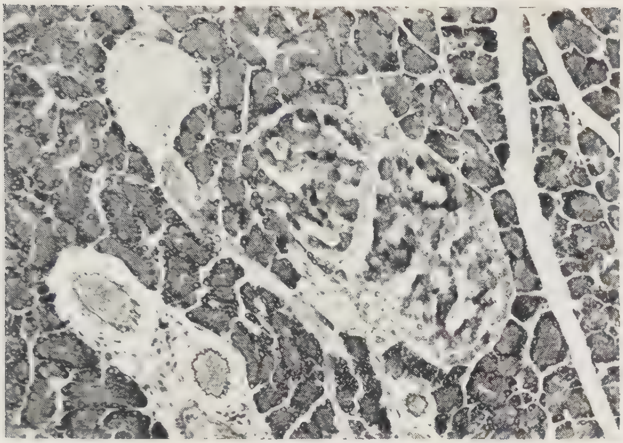

Fig. 2.
Typical pancreatic islet from rat II-3 which died after only 10 days of force feeding. Note the almost normal appearance of the islet cells. Chrome-hematoxylin and phloxine stain. × 150.

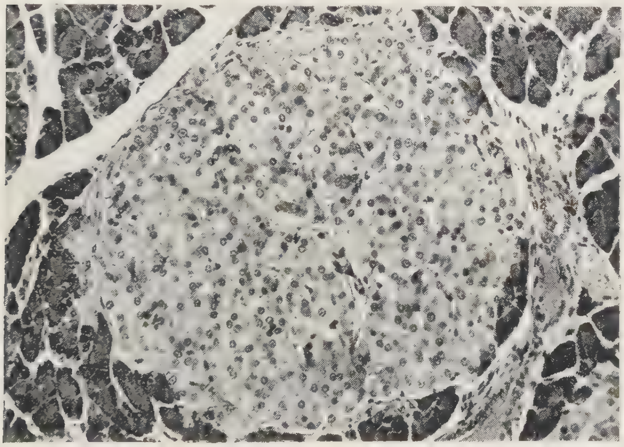

Fig. 3.
Typical pancreatic islet from rat II-1 which was sacrificed after 56 days of force feeding. Note the marked hyperplasia of the islet cells with almost complete degranulation of the beta cells. Chrome-hematoxylin and phloxine stain. × 150.

changes. Although the pitfalls of evaluating islet volume from examination of a single section through the pancreas are well established,[10] these hyperplastic changes were so uniform and conspicuous that there is little doubt of their significance. Furthermore, the recent study of Tejning[11] gives us added confidence in the validity of our interpretation. After detailed studies of pancreatic islet volumes, this investigator concluded that if one finds in a section of pancreas a number of islets that are larger than normal, one can conclude that the total volume of islet tissue is also greater than normal. No evidence of necrosis, hydropic change, hemorrhage, inflammation or absolute decrease in beta cells was apparent in any of the sections.

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[10] Richardson, K. C., and Young, F. G., *J. Physiol.*, 1937, **91**, 352.

[11] Tejning, S., *Acta Med.* Scandinav. Supp., 198, 1947.

Other significant histologic findings consisted of a somewhat variable but often marked increase in glycogen in the liver cord cells, sometimes accompanied by slight large droplet fatty change. The stomachs of the rats which had been force fed for a period of 30 days or more showed a spectacular hypertrophy of the muscularis but no other abnormality. The thymus glands showed microscopic evidence of atrophy in only 3 rats, and this was in no instance well developed. The other organ showing rather consistent alteration was the adrenal. Here there was a moderate cortical hyperplasia which was evident in 7 rats. There was no apparent correlation between the adrenal cortical hyperplasia and the thymic atrophy.

The kidneys as well as the skeletal muscle and the structures of the mediastinum revealed no significant histologic changes in the instances in which they were examined. Unfortunately, the testes were studied microscopically in only one rat (number 4, Group I). Here the tubules were of normal size and cellularity and spermatogenesis was apparently active.

*Discussion.* Our data are not complete enough to warrant conclusions to be drawn regarding the adrenal cortical hyperplasia. The similarly treated rats of Ingle[7] uniformly showed grossly enlarged, dark red, adrenal cortices. The findings may be due to the very large amounts of carbohydrate administered, to the stress incident to the acts of force feeding, or to some other factor. High protein diets have been reported to cause adrenal cortical enlargement,[12] but the protein contained in the diets fed to our rats probably was not sufficient at any time to cause such changes.

The concentration of sugar in the blood has repeatedly been found to affect islet structure and volume. The islet cell degeneration produced by partial pancreatectomy and/or anterior pituitary extract injections in the dog and cat can be prevented or, in certain instances, reversed by a low carbohydrate intake, insulin, or phloridzin.[1-4,13] Rabbits treated with alloxan develop hydropic degeneration of the islets after about one month of marked hyperglycemia.[14] Corpaci believed that he had increased islet volume and produced damage of islet cells in rabbits by repeated parenteral injections of glucose and adrenalin, but the evidence is not conclusive.[15] Similar experiments in guinea pigs by Meneghini, using glucose alone, are reported to have caused islet hyperplasia.[16] Careful studies have shown that rats fed a high carbohydrate diet *ad libitum* develop pancreatic islet volumes that are significantly greater than those fed *ad libitum* other types of diets.[11] Evidence has been obtained of both hyperplasia and exhaustion of guinea pig islets after continuous infusions of large amounts of glucose.[17,18] In experiments lasting not more than 100 hours, dogs killed by the continuous intravenous administration of glucose were found to have specific intense hemorrhages into and destruction of the pancreas and hypophysis.[19] Special studies of islets were not reported. Finally, clinical diabetes has been produced in an intact cat after 39 days of intraperitoneal glucose injections, and part of a series of cats treated in this way developed marked hydropic degeneration of the islets.[5,6] Verzár and von Kúthy produced heavy glycosuria, mild hyperglycemia, and, on rare occasions, ketonuria in dogs by force feeding solutions of glucose and sucrose for several weeks.[20] Glucose tolerance was thereby impaired but soon returned to normal. Postmortem examinations are not recorded.

Degeneration of rat pancreatic islets has not been produced by hyperglycemia. Lukens and Dohan examined the pancreatic remnants of rats that had had pronounced hyperglycemia for from 2 weeks to several months following partial pancreatectomy and found almost no damage.[21] In similarly treated animals hyperplasia instead of damage was reported by Friedman and Marble.[22] The same type of change was observed after the administration of anterior pituitary extract.[10] In order to produce glycosuria consistently in the rat with anterior pituitary extract, partial pancreatectomy is necessary,[23] although adrenal steroids cause temporary insulin resistant diabetes.[24] However, Peterson has recently reported complete degranulation of rat islet beta cells within 15 minutes of the intracardiac injection of 3.0 g of glucose/kg.[25] This lasts 48 hours and is associated with temporary impairment of glucose tolerance. Similar but less consistent degranulation was found after one intraperitoneal injection of glucose in the guinea pig by Gomori *et al.*[26]

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[12] Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinol.*, 1943, **32**, 403.

[13] Lukens, F. D. W., Dohan, F. C., and Wolcott, M. W., *Endocrinol.*, 1943, **32**, 475.

[14] Duff, G. L., McMillan, G. C., and Wilson, D. C., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 251.

[15] Corpaci, A., *Sperimentale, Arch. di biol.*, 1932, **86**, 129.

[16] Meneghini, T., *Ginecologia*, 1939, **5**, 539.

[17] Woerner, C. A., *Anat. Rec.*, 1938, **71**, 33.

[18] Woerner, C. A., *Anat. Rec.*, 1939, **75**, 91

[19] Jacobs, H. R., and Colwell, A. R., *Am. J. Physiol.*, 1936, **116**, 194.

[20] Verzár, F., and von Kúthy, A., *Pflueger's Arch. f. d. gesam. Physiol.*, 1930, **225**, 606.

In canine anterior pituitary diabetes, proliferation of the islet cells may precede the exhaustive or degenerative changes which accompany the onset of permanent diabetes.[27] Our observations indicate that the carbohydrate tolerance increased with each increase in feeding, glycosuria decreasing when the diet was maintained at a level which had initially caused moderate sugar excretion. This adaptation is evidently implemented by islet hyperplasia. Although it might be possible to prevent hyperplasia and produce islet damage by increasing the volume of food more rapidly this would probably be difficult without causing fatal "food-shock." It is conceivable that some relatively small additional stimulus, perhaps the administration of anterior pituitary extract or adrenal steroids, might have produced exhaustion of the islets of our rats. Older rats may be more susceptible.[28]

*Summary.* Degranulation of beta cells of the pancreatic islets and islet hyperplasia have been observed in rats which were force-fed large amounts of high carbohydrate, otherwise balanced diets for periods of as long as 8 weeks. Glycosuria and, in rare instances, ketonuria occurred.

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[21] Lukens, F. D. W., and Dohan, F. C., *Endocrinol.*, 1942, **30**, 175.

[22] Friedman, N. B., and Marble, A., *Endocrinol.*, 1944, **29**, 577.

[23] Long, C. N. H., *Harvey Lect.*, 1936-37, **32**, 194.

[24] Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinol.*, 1945, **37**, 341.

[25] Peterson, C. A., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 352.

[26] Gomori, G., Friedman, N. B., and Caldwell, D. W., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 567.

[27] Richardson, K. C., and Young, F. G., *Lancet*, 1938, **1**, 1098.

[28] Martinez, C., *Rev. Asoc. med. argent.*, 1946, **60**, 666.

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## 17175. Inorganic Aging of the Plasma Layer of Tissue Cultures.

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The problem of maintaining mammalian cells *in vitro* for long intervals in an original or lightly patched plasma base may be broken down into two aspects: (a) the avoidance of calcification during the usual transfer intervals of two to four weeks and (b) the problem of an inorganic aging which complicates more prolonged maintenance. Though the control of calcification difficulties during the usual transfer intervals seems feasible by the measures summarized in the preceding paper,[1] solution of the long-term problems arising from inorganic aging appears more formidable.

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[1] Hanks, J. H., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 328.

It is a classical observation that even in the absence of evident calcification the plasma layer of cell cultures gradually becomes less and less favorable to cell growth. Simms and Stillman[2] have presented evidence that tryptic digestion removes from adult tissues and from the plasma of cell cultures an inhibitor having some of the properties of calcium phosphate. It is also known that inorganic gels undergo changes in viscosity, pH, extinction coefficients, and other physical properties during aging. These changes can be explained in part by preferential liberation and adsorption of ions, that is by ionic exchange.[3] Particularly in circumstances where a gel is in contact with supernatant fluids which are changed repeatedly, the concentration of preferentially adsorbed ions will become radically different from the ionic analysis at the moment the gel is formed.[4] Such procedures are an inherent feature of the long-term tissue culture technics.

It is the purpose of the present communication to present evidence concerning the adsorption of calcium by the plasma layer of tissue cultures and the difficulty of decalcifying this substrate. Methods have been summarized in the preceding paper.[1]

*Experimental observations.* The firmness with which calcium and phosphates are adsorbed to the plasma layer became apparent as the result of attempts to remove opacity from cell colonies and plasma which had become calcified. This opacity could not be diminished by using supernatant media prepared by diluting the serum and embryo juice in salt solution lacking both Ca and P or by prolonged depression of pH during cultivation within physiological limits of H ion concentration. Incorporation of 0.1 to 0.2% sodium citrate in media of minimal Ca and P levels or in mildly acidified washing solutions killed the cells without clearing the plasma. Direct extraction with N/100 HCl in the balanced salt solution or weaker acids in the balanced salt solution likewise failed to clear the plasma before the cells were irreparably damaged. The deposits were rapidly removed by N/10 HCl, which was fatal to cells. Optimal cell survival followed rapid clearing of the plasma in N/40 HCl in the salt solution for 5 to 20 minutes as required. Since the complicated procedure required to effect this result and obtain prompt neutralization without cell damage is not to be recommended,* these observations are summarized only in order to illustrate the fact that the Ca adsorbed by the plasma is not brought into solution by complex-forming anions such as citrate and is liberated only by ionic exchange with fairly high concentrations of H ion.

Other evidence of the role of ionic exchange in the inorganic aging of plasma was obtained from data on the increased deposition of Ca in the plasma layer during repeated renewal of cultures with supernatant fluids which do not produce calcification within the usual transfer intervals. After 6 weeks of cultivation in media composed of human serum 40% and beef embryo juice 4% diluted in the salt solution containing Ca 5 mg% and P 2.2 mg% (six renewals) the slightly opalescent plasma layers of 10 cultures were rinsed and extracted with N/10 HCl. Analysis revealed that this plasma contained from 24 to 40 mg% of Ca, *i.e.*, three to five times the original concentration of Ca in the plasma.

When the same formula (sera 40%, embryo

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[2] Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, **20**, 603.

[3] Hanks, J. H., and Weintraub, R. L., *J. Phys. Chem.*, 1937, **41**, 583.

[4] Jenny, H., *J. Phys. Chem.*, 1932, **36**, 2217.

* A more practical procedure is the complete removal of opaque plasma by digestion with 0.5% trypsin or pancreatin in the salt solution at pH 7.8. This process must be watched carefully and stopped before the naked cells, which retract around the explants, are washed away (all liberated fibroblasts appear to be damaged). Since digestion is never completed simultaneously in all cultures, the simplest procedure is to replace the digestion mixture in each culture with two drops of 50% plasma at the appropriate moment, to exhale alveolar air to depress the pH, and to stopper quickly. The liquid plasma should be rolled around the culture vessels several times and then be allowed to cover the explants and establish a bond with the wet glass before the third drop of medium or coagulant is added to induce simultaneous coagulation in all cultures thus accumulated.

juice 4%) is prepared in salt solution lacking both Ca and P, it contains approximately one half the usual levels of these elements. Though this solution delays the darkening of the central portion of transplants, there is no deficit in calcium compounds, and the central transplants eventually become opaque to transmitted light. This solution is superior to the usual formula for prolonged maintenance of cells in a plasma base.

*Discussion.* The demonstration of a 5-fold increase of Ca ions in a plasma base following serial renewals of a supernatant fluid, and of the necessity of hydrogen ions for its replacement, indicates that the plasma substrate in cell cultures is the site of an ionic exchange. A more complete analysis could be expected to show that not only Ca but also other preferentially adsorbed ions tend to replace less tightly bound but physiologically important ions. It may be inferred that cells maintained in an original or lightly patched plasma base spend a considerable portion of their existence in an inorganic environment quite different from what is intended and that the inorganic composition of renewal supernates might be improved by altering radically the ionic balance now normally present in fluids prepared from complex nutrients. It is also evident that existing data on the role of different ions in plasma cultures may require re-evaluation by culture methods which expose the cells directly to the fluids being studied.

The present data would appear to confirm the observations of Simms and Stillman[2] on the nature of at least one of the inhibitors which occur in plasma and adult tissues and is capable of being removed by tryptic digestion.

*Summary.* The adsorption of calcium phosphate complexes to plasma substrates, and the conditions required for their removal, indicate that the plasma gel is the site of an ionic exchange in which preferentially adsorbed ions may greatly alter the desired ionic balance. This process is one of the factors which causes plasma to become growth inhibitory during prolonged maintenance of cells in this type of substrate.

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## 17176. Tolerance of Normal and Partially Depancreatized Rats for Insulin.

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It was shown by this study that the average lethal dose of insulin is smaller in force-fed depancreatized rats than in normal rats.

*Methods.* Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets prior to the beginning of the experiments. At a weight of 310-320 g, some of the animals were subjected to a single-stage pancreatectomy[1] in which all but tiny remnants lying between the duodenum and bile duct were removed. After the rats recovered from the operation, they were placed in metabolism cages and were adapted to the force-feeding[2] of a medium carbohydrate diet (Table I) in amounts of 13 cc each morning and late afternoon. The urines were collected at 8:00 to 8:30 A.M. each day and were preserved by thymol. Urinary glucose was determined by the method of Benedict.[3]

*Experiments and results.* Severely diabetic rats were selected for these experiments. The average amount of glucose excreted by the 18 depancreatized rats used in this study was 6096 mg per day. The amount of glucose es-

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[1] Ingle, D. J., and Griffiths, J. Q., Jr., Chapter 16, The rat in laboratory investigation. J. B. Lippincott Co., Philadelphia, 1942.

[2] Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 44.

[3] Benedict, S. R., *J.A.M.A.*, 1911, **57**, 1193.

TABLE I.
Medium Carbohydrate Diet.

| Constituent | g |
|---|---|
| Cellu flour (Chicago Dietetic Supply) | 120 |
| Osborne & Mendel salt mixture | 40 |
| Dried yeast (Pabst) | 100 |
| Wheat germ oil | 10 |
| Cod liver oil | 10 |
| Vit. K (2-methyl-1,4-naphthoquinone) | 100 mg |
| Mazola oil | 200 |
| Casein (Labco) | 160 |
| Starch | 200 |
| Dextrin | 190 |
| Sucrose | 200 |
| Water to make total of | 2000 cc |

TABLE II.
Lethal Dose of Regular Insulin Expressed as Units for Rat per Day. Means and standard deviations of the means and differences.

| | No. | Mean | Difference |
|---|---|---|---|
| Normal | 20 | 68.5 ± 1.77 | |
| Depancreatized | 18 | 50.5 ± 4.27 | 18 ± 4.68 |

timated to be available from the carbohydrate, glycerol and protein (assuming 60% conversion of protein to glucose) of the diet is approximately 6500 mg per rat per day. These severely diabetic rats were then compared to normal force-fed rats in respect to the amount of insulin required to kill the animal. Regular insulin (Lilly) was administered by subcutaneous injection immediately following each feeding. The initial dose was 5 units given twice each day. The dosage was increased by 5 units per injection on every second day until the animal died.

The data are summarized in Table II. Normal rats tolerated greater average amounts of insulin than did the diabetic rats. The variability among individual animals was greater among the depancreatized rats than among the normal animals. The difference between the means for the two groups is highly significant from the statistical standpoint.

*Discussion.* If it were assumed that the secretion of insulin is unaltered by the administration of exogenous insulin, it would be expected that a lethal level of insulin dosage would be reached more rapidly in the normal animal than in the depancreatized animal. It seems probable from these and other data[4] that the secretion of insulin by the pancreatic islets is inhibited by the administration of an excess of exogenous insulin. Even this assumption does not explain the increased sensitivity of the depancreatized animal to insulin. It has been noted that totally depancreatized patients[5] may show greater sensitivity to insulin than the average patient with diabetes mellitus. Young[6] reported that pancreatectomy of a dog with pituitary diabetes lowered its insulin requirement. Dragstedt *et al.*[7] noted that partially depancreatized dogs may have a higher insulin requirement than totally depancreatized dogs. Thorogood and Zimmerman[8] found that dogs having alloxan diabetes showed a decrease in insulin requirement following pancreatectomy. Howard[9] has commented upon "The almost uniform finding that an organism is highly oversensitive to a hormone that it lacks, and the corollary that the normal is relatively insensitive to doses of a hormone which would seriously injure the deficient animal. . . one wonders if the gland itself may not sometimes reverse its processes, and, in the presence of an administered excess of its hormone, actually begin to destroy it or elaborate some counteracting hormone." A substance which causes glycogenolysis and hyperglycemia has been obtained from extracts of pancreatic islets[10] and has been considered as a possible antagonist of insulin but it is not known if it is a normal secretory product of the pancreas or if it has any physiological role.

An additional explanation of these results could be based upon the assumption that the assimilation of food was incomplete due to a deficiency in the external secretion of the pancreas and that fatal hypoglycemia developed more rapidly for this reason. There was no diarrhea in these experiments, the feces were well formed and firm but were larger than normal in the depancreatized rats. The completeness of intestinal absorption was not studied. Similarly, it is possible that the depancreatized rats were debilitated by operation and by the period of uncontrolled glycosuria so that their resistance to insulin shock was lower than normal. Although the depancreatized animals appeared vigorous and healthy during the period of insulin administration, it would be unsafe to conclude that their lower than normal resistance to insulin was due solely to the absence of the pancreatic islets.

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[4] Haist, R. E., *Physiol. Rev.*, 1944, **24**, 409.

[5] Waugh, J. M., Dixon, C. F., Clagett, O. T., Bollman, J. L., Sprague, R. G., and Comfort, M. W., *Proc. Staff. Meet. of the Mayo Clinic*, 1946, **21**, 25.

[6] Young, F. G., *New England J. Med.*, 1939, **221**, 635.

[7] Dragstedt, L. R., Allen, J. G., and Smith, E. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 292.

[8] Thorogood, E., and Zimmerman, B., *Endocrinology*, 1945, **37**, 191.

[9] Howard, J. E., *American Practitioner*, 1948, **2**, 674.

[10] Heard, R. D. H., Lozinski, E., Stewart, L., and Stewart, R. D., *J. Biol. Chem.*, 1948, **172**, 857.

*Summary.* Eighteen extensively depancreatized male rats and 20 normal rats were force-fed a medium carbohydrate diet. Following periods of adaptation and control, all of the animals were tested for their resistance to twice daily injections of regular insulin. The initial dose was 5 units and was increased in increments of 5 units per dose every second day until the animal died. The average lethal dose for the depancreatized rats was 50.5 units per 24 hours as compared to an average of 68.5 units for the normal animals. This difference in averages was calculated to be statistically significant.

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## 17177. Isolation of Three Strains of Type B Influenza Virus Incompletely Related to Lee.

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During December 1948, reports of widespread distribution of influenza began to appear in the Seattle area. The majority of illnesses were of relatively short duration and were accompanied in many cases by gastrointestinal disturbances. Proper specimens of throat washings and serum were not obtainable until the return of students to the University of Washington campus after the New Year holidays.

During the first 9 weeks of 1949, there were 116 admissions for upper respiratory infection to the University of Washington Health Center. It was apparent, through random questioning of the campus population, that many others were ill at home. Since approximately 60% of the 16,000 University students live at home in or close to the City of Seattle, and there were a number of students known to be ill in living quarters on and off the campus, it seems obvious that the outbreak was much more widespread than the 116 admissions to the Health Center would indicate. A conservative estimate of 500 cases, of varying severity, during the period from the end of December to the first of March does not seem unreasonable. Observations of classroom attendance supported this estimate.

The majority of illnesses were mild, with the average period of hospitalization being between 3 and 4 days. The mildness of the disease further supports the idea that the cases seen in the campus infirmary represented only a relatively small proportion of the total number occurring in the Seattle area.

*Materials.* Blood specimens and throat washings were obtained during the acute stage from patients in the infirmary whenever possible. Serum was separated promptly and stored at –2°C until used. Throat washings were obtained by having the subjects gargle 15 ml of nutrient broth (Bacto) to which had been added penicillin G in an approximate concentration of 1000 units per ml. The garglings were collected in clean beakers, transferred to sterile screw cap bottles, and stored in a $CO_2$ ice box at approximately –76°C until used.

Convalescent blood specimens were obtained whenever possible. The interval between the acute and convalescent specimens ranged from 10 to 25 days, with a few exceptions. Twenty-eight convalescent sera were obtained from suspected influenza cases, and were stored with the acute specimens at –2°C.

*Methods.* Throat washings were inoculated to the allantoic cavity of 8- to 10-day chick embryos, using 0.25 ml per egg and a minimum of 5 eggs per washing. Inoculated eggs were incubated at 36°C and were examined daily by candling. Eggs showing dead embryos in 24 hours were discarded. A small portion of allantoic fluid was aspirated daily from surviving eggs from the second to fifth days after inoculation, and was tested in a 1 to 10 dilution for ability to agglutinate washed human cells of group O. Fluids showing typical agglutination were inoculated to a new set of eggs and were further tested against standard antisera in an attempt to determine the type of virus most prevalent in the outbreak.

The technic for examination of paired sera was that recommended by the Influenza Information Center of the U. S. Public Health Service[1] for laboratories participating in its program. The original method,[2] as modified by the Army Medical Center,[3] involves the addition of 4 hemagglutinating units of the standard virus antigens (PR8, FM1 and Lee) to serial two-fold dilutions of inactivated serum, followed by addition of washed group O human red cells. Incubation was for one hour at room temperature.

*Results.* Three strains of influenza virus were isolated during the early part of the outbreak, and gave typical agglutination results up to 1 in 320, in the first passage. Subsequent allantoic passages yielded fluids with titers ranging up to 1 in 2560. The strains were designated as Seattle 1-49, 2-49, and 3-49, and were forwarded to the Influenza Strain Study Center.[4]

The 3 virus strains were separately tested by the standard agglutination-inhibition technic against high-titer monovalent rooster sera.* These sera were able to inhibit red cell agglutination by PR8, FM1 or Lee antigens in titers of 1 in 1600 or 1 in 800, with cross-inhibition only in the first tube between the A and A prime viruses. However, when the 3 newly isolated strains of influenza virus were set up against the standard sera, no significant evidence of inhibition of hemagglutination could be obtained. Additional egg passages up to a total of 10 did not modify these results. The specific rooster sera were not able to supply the typing information desired, and because of doubt regarding the nature of the prevalent virus, a general program of vaccination was not undertaken.

Twenty-eight paired sera were investigated for evidence of rise in antibody titer against types A, A prime and B viruses, and also against the Seattle strains, using the standard agglutination-inhibition technic. No pairs showed any evidence of significant increase against type A or A prime viruses and these data are therefore omitted. The results of the significant titrations are shown in Tables I and II.

Table I gives the results of titrations on 28 paired sera. Six patients showed no increase of antibodies able to inhibit hemagglutination by known virus antigens and were not considered as serologically proved cases. The titrations of the 22 sera showing a significant increase of such antibodies gave the results indicated. It will be noted that the evidence indicates a higher antibody rise against Lee virus than against the Seattle strains.

The difference in antigenicity between the Lee and Seattle viruses is demonstrated further in Table II. It is seen that of the 22 serum pairs showing a 4-fold or greater increase in titer against Lee virus, only 18 also showed

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[1] Culbertson, James T., *Am. J. Pub. Hlth.*, 1949, **39**, 37.

[2] Anonymous, *Bull. U. S. Army Med. Dept.*, 1946, **6**, 777.

[3] Medical Division, Army Medical Center, letter of Sept. 7, 1948.

[4] Anonymous, *Science*, 1947, **106**, 542.

* Obtained through the kindness of the Department of Virus and Rickettsial Diseases, Army Medical Center, Washington, D.C.

TABLE I.
Titrations of Paired Sera.

| Increase | 4-fold | 8-fold | 16-fold or more | Total |
|---|---|---|---|---|
| vs. Lee | 6 | 7 | 9 | 22 |
| vs. Seattle strains | 9 | 8 | 1 | 18 |
| No significant increase in titer | | | | 6 |

TABLE II.
Increase in Titer of Paired Sera.

| Significant increase | |
|---|---|
| vs. both Lee and Seattle strains | 18 |
| vs. Lee only | 4 |
| vs. Seattle strains only | 0 |
| No increase | 6 |
| Total | 28 |

a significant rise against the Seattle strains. Four patients showing a rise in titer of antibodies able to inhibit hemagglutination by Lee virus and not by the Seattle strains, may represent cases due to viruses other than the more prevalent strains.

On the basis of titrations of paired sera only, the outbreak in Seattle would be considered as due to a virus antigenically related to Lee virus, since all the positive sera showed a significant increase in antibodies against Lee when compared with the acute phase sera. On the other hand, hemagglutination by the Seattle viruses was not inhibited by typing sera prepared in roosters. This fact emphasizes two points: first, an antigenic difference between the newly isolated strains and Lee virus, and second, the inadequacy of highly specific antisera for the typing of influenza virus having minor antigenic differences from the standard strains.

Because of the ability of Seattle viruses to produce antibodies against Lee virus, and the failure of specific Lee antiserum to inhibit hemagglutination by the new strains, it is apparent that the Seattle strains are not antigenically identical with Lee virus. This opinion has been confirmed by the virus laboratory of the California State Department of Health[5] and by the Influenza Strain Study Center.[6]

The evidence in the case of the Seattle strains seems to indicate that these viruses are not as highly antigenic as Lee virus, as demonstrated by the lesser rise in titer in paired sera, but that the new strains may be antigenically broader in pattern, as shown by the ability of all second sera to inhibit hemagglutination by Lee significantly, whereas specific Lee serum did not inhibit hemagglutination by the new strains.

Strain differences in influenza B virus have been reported previously by Eaton and Beck,[7] Gordon,[8] Burnet *et al.*,[9] and others. However, it is considered of value to record the continued antigenic heterogeneity of this virus, particularly in view of the problem of choice of strains for inclusion in influenza vaccines.

*Summarizing*, there is reported an outbreak of influenza on the University of Washington campus, from which 3 strains of influenza virus B were isolated. Paired sera from 22 patients showed a significant rise in hemagglutination-inhibition titer against Lee virus in all cases and against the new strains in 18 sera. Because of the inability of rooster antiserum prepared against Lee virus to inhibit hemagglutination by the Seattle strains, it appears that these new viruses are incompletely related to the standard type B virus.

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[5] Meiklejohn, G., personal communication, Feb. 11, 1949.

[6] Magill, T. P., personal communication, March 18, 1949.

[7] Eaton, M. D., and Beck, M. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 177.

[8] Gordon, I., *J. Immun.*, 1942, **44**, 231.

[9] Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Aust. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 9.

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# 17178. Some Reactions of the Rat to Treatment with Progesterone and Estrone in Late Pregnancy.*

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Following the observation that extract of corpora lutea[1] prolonged pregnancy in the rat, reports on the effects of estrogens[2-4] and progesterone[4,5] on parturition in the rat have appeared. These reports have not included observations on the behavior of the treated animals at the normal time for delivery. Because pregnancy was allowed to continue the viability of the young at the normal time for delivery was not determined. The purpose of the present study is to observe viability of the young and labor in rats treated daily during late pregnancy with estrone, progesterone or a combination of estrone and progesterone.

*Methods.* In the strain of rats (Anheuser-Busch) used in these experiments the young were normally delivered in the night following the 21st day of pregnancy or in the morning of the 22nd day. The day that sperms were observed in the vaginal smear was counted as the first day of pregnancy. The hormone injection was continued daily from the time of the first injection. The young were removed in the afternoon of the 22nd day by hysterectomy in animals that had not delivered spontaneously. The passage of blood from the vagina was regarded as evidence of uterine bleeding. Confirmatory evidence of uterine bleeding was obtained when the young were removed.

*Results.* A summary of the results obtained is shown in Table I. Although uterine bleeding occurred in the 3 animals given 1 mg of progesterone, attempts to deliver the young were seen in only 1. The other 2 rats were quiescent and contraction of abdominal muscles observed prior to parturition in normal animals was not seen. At the time of removal 20% of the young were dead. No labor attitudes or uterine bleeding occurred in 4 animals given 2.5 mg of progesterone and these animals remained quiet and showed no evidence of efforts to deliver the young. Only 3% of the young were dead at the time of removal.

The well-known tendency of estrogen to terminate pregnancy led us to attempt to bring about spontaneous delivery of young in late pregnancy. The data in Table I indicate that administration of estrone in late pregnancy may either prevent parturition or accelerate delivery. It was found that daily injection of 25 $\gamma$ of estrone started on the 19th or 20th day of pregnancy caused prolonged labor but failure to deliver the young by the end of the 22nd day. One animal given 100 $\gamma$ of estrone on the 20th day delivered young 1 day premature less than 20 hours after the injection. In 4 animals treated with 25 $\gamma$ of estrone about 24 hours before time for delivery parturition seemed to be accelerated rather than prevented; about 12 to 18 hours after the first injection the animals became extremely active and delivered the young promptly. A point of particular interest is the sharp contrast in behavior of the estrone-treated animals as compared with the ones given progesterone. Those estrone-treated animals requiring removal of the young showed vigorous and prolonged efforts to deliver spontaneously. In the rats treated with estrone 40% of the young were dead on the 22nd day of pregnancy.

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* Aided by a grant from the U. S. Public Health Service.

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[2] Hain, A. M., *Quart. J. Exp. Physiol.*, 1935, **25**, 131.

[3] D'Amour, F. E., and Dumont, C., *Quart. J. Exp. Physiol.*, 1937, **26**, 215.

[4] Boe, F., *Acta path. et Microbiol. Scandinav.*, suppl. 36, 1938.

[5] Imaz, F. A. U., *Prensa Méd. Argent.*, 1943, **30**, 2309.

TABLE I.
Effects of Progesterone and Estrone in Late Pregnancy in the Rat.

| Rat No. | Treatment daily* | Day begun | Delivery | Fetuses | | Remarks |
|---|---|---|---|---|---|---|
| 31 | 1 mg P† | 19 | Removed§ | 5 living, | 4 dead | No labor. Uterine bleeding |
| 32 | 1 mg P | 19 | ,, | 11 ,, | | ,, ,, ,, ,, |
| 35 | 1 mg P | 19 | ,, | 4 ,, | 1 ,, | Labor. Uterine bleeding |
| 42 | 2.5 mg P | 19 | ,, | 10 ,, | | No labor. No uterine bleeding |
| 44 | 2.5 mg P | 19 | ,, | 9 ,, | | ,, ,, ,, ,, ,, |
| 45 | 2.5 mg P | 19 | ,, | 7 ,, | | ,, ,, ,, ,, ,, |
| 46 | 2.5 mg P | 19 | ,, | 9 ,, | 1 ,, | ,, ,, ,, ,, ,, |
| 50 | 200 $\gamma$ E‡ | 19 | Autopsy | | 8 ,, | Died 24th day after prolonged labor |
| 51 | 25 $\gamma$ E | 19 | Removed | 2 ,, | 5 ,, | Labor. Uterine bleeding |
| 55 | 100 $\gamma$ E | 20 | Spontaneous | 11 ,, | | 1 day premature |
| 56 | 25 $\gamma$ E | 20 | Removed | 1 ,, | 6 ,, | Labor. Uterine bleeding |
| 63 | 25 $\gamma$ E | 21 | Spontaneous | 3 ,, | 3 ,, | 22nd day of pregnancy |
| 64 | 25 $\gamma$ E | 21 | ,, | | 7 ,, | ,, ,, ,, ,, |
| 65 | 25 $\gamma$ E | 21 | ,, | 7 ,, | | ,, ,, ,, ,, |
| 66 | 25 $\gamma$ E | 21 | ,, | 11 ,, | | ,, ,, ,, ,, |
| 71 | 25 $\gamma$ E | 18 | ,, | 4 ,, | 5 ,, | Parturition delayed 1 day. Prolonged labor |
| 81 | 100 $\gamma$ E + 1 mg P | 19 | Removed | 7 ,, | 2 ,, | No labor. Uterine bleeding |
| 82 | 100 $\gamma$ E + 1 mg P | 20 | ,, | 8 ,, | | ,, ,, ,, ,, |
| 92 | 25 $\gamma$ E + 2.5 mg P | 20 | ,, | 10 ,, | | No labor. No uterine bleeding |
| 93 | 25 $\gamma$ E + 2.5 mg P | 20 | ,, | 8 ,, | | ,, ,, ,, ,, ,, |
| 1-5 | None | — | Spontaneous | 43 ,, | 2 ,, | 22nd day of pregnancy |

* Except in rat No. 71, which received single injection.

† P = Progesterone.

‡ E = Estrone.

§ In all animals except Nos. 50 and 71, the young were delivered by hysterectomy toward end of 22nd day of pregnancy, if young were not delivered spontaneously.

The rats injected simultaneously with estrone and progesterone showed no effort to deliver the young and remained relatively inactive. It is possible, however, that labor may have occurred in some of the animals during the night when the animals were not under observation. Uterine bleeding was seen only in the 2 animals given large amounts of estrone. Only 6% of the young were dead.

*Discussion.* In only 2 studies of which we are aware has the daily injection of crystalline progesterone been continued in the rat beyond the normal term of pregnancy. Pregnancy was prolonged by daily injection of 1 mg of progesterone started the 18th day of pregnancy[4] and by 0.5 mg daily started on the 16th day.[5] The observation[6] that 2 Corner-Allen Rb. U. (2 mg) of progesterone are required to maintain pregnancy in the ovariectomized rat suggested to us that the dosages of this hormone used in the above studies on parturition may not completely prevent labor. In the rabbit, for example, it has been shown that 1 mg of progesterone maintains pregnancy after removal of the ovaries[7] while 1.5 mg is required to prolong pregnancy significantly.[8] Our results show that 2.5 mg of progesterone daily are required to prevent uterine bleeding when injections are begun on the 19th day of pregnancy.

It has been shown[4] that 600 $\gamma$ and 300 $\gamma$ of estrone benzoate given daily from the 16th to the 22nd day of pregnancy in the rat kills most of the young and delays delivery. Similar results[2] were obtained in the rat with daily injection of 125 $\gamma$ to 500 $\gamma$ of estrogens begun late in pregnancy. Heckel and Allen[9] found that during daily injection of estrone in pregnant rabbits beginning on the 29th day 400 I.U. killed the young but did not significantly delay parturition and 1000 I.U. prevented delivery; delivery occurred normally on the 32nd day in this strain of rabbits. The prevention of parturition in the rabbit was regarded as due primarily to prolongation of the functional state of corpora lutea. The prolongation of pregnancy in the hypophysectomized rabbit[10] and in the rat[11] by treatment with estrogens was similarly interpreted. Our findings that estrone injection started on the 19th day of pregnancy in the rat brought about vigorous but ineffective efforts to deliver young while progesterone treatment started on the same day induced quiescence, suggest that estrone plays a role in the prevention of parturition in the rat other than by way of its action on the corpora lutea. Because estrone apparently increases the frequency and intensity of contraction of abdominal muscle, it is suggested that failure to deliver the young is due to obstruction of the birth canal possibly as a result of failure of relaxation in the region of the cervix. In the rat wide abdominal incision during parturition practically stops transport and expulsion of the fetuses and closure of the incision restores both processes.[3] This observation, which was interpreted as evidence that contractions of the diaphragm and abdominal muscles provide the primary force for delivery of young, supports our view that failure of expulsion in estrone-treated rats showing vigorous labor is due to failure of uterine relaxation.

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[6] Rothschild, I., and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 402.

[7] Allen, W. M., and Corner, G. W., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 403.

[8] Heckel, G. P., and Allen, W. M., *Am. J. Physiol.*, 1937, **119**, 330.

[9] Heckel, G. P., and Allen, W. M., *Endocrinology*, 1939, **24**, 137.

Our observation that progesterone injection started simultaneously with estrone treatment on the 19th or 20th day of pregnancy prevents labor, uterine bleeding and death of young suggests that the death of fetuses induced by estrone may be due, at least in part, to intense but ineffective labor.

*Summary.* Progesterone (2.5 mg daily) beginning on the 19th day of pregnancy in intact rats prevents labor, uterine bleeding and parturition. Estrone (25 $\gamma$) started on the 19th or 20th day induces uterine bleeding, vigorous but ineffective labor and death of fetuses. Estrone (100 $\gamma$) begun on the 20th day in 1 animal brought about delivery of young 1 day premature. Spontaneous delivery occurred in 4 rats given 25 $\gamma$ of estrone about 24 hours before time for delivery. Progesterone (2.5 mg) given simultaneously with estrone (25 $\gamma$) beginning on the 20th day has the same effect as progesterone alone, preventing the above effects of estrone alone. It is suggested that estrone plays a role in the prevention of parturition in the rat other than by way of its action on the corpora lutea.

Progesterone from Schering Corporation, Bloomfield, N. J., and estrone from Parke Davis and Co., Detroit, Mich., were generously supplied for this study.

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[10] Robson, J. M., *J. Physiol.*, 1940, **97**, 517.

[11] Odendaal, W. A., *South African J. M. Sc.*, 1944, **9**, 131.

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## 17179. Urinary Excretion of Phosphate Following the Injection of Sodium p-Aminohippurate.

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In the course of recent studies of osmotic diuresis in man,[1] the rate of urinary excretion of phosphate was found to be increased following the injection of sodium p-aminohippurate. Although PAH has been found to

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* Senior Fellow in Pediatrics of the National Research Council. Aided by a grant from the National Foundation for Infantile Paralysis.

[1] Rapoport, S., West, C. D., and Brodsky, W. A., *Am. J. Physiol.*, in press.

TABLE I.
Effect of NaPAH on Urinary Phosphate Excretion.

| Period No. and specimen | | Concurrent time, min. | Urine volume, cc/min./1.73 $M^2$ | PAH conc., mg % | Phosphate conc., m.Osm/L | Phosphate excreted, m.Osm/min./1.73 $M^2$ |
|---|---|---|---|---|---|---|
| L.J. Hydration. 9 yrs. S.A. 1.25 $M^2$. Wt. 37.3 kg. | | | | | | |
| P–1 | U | –66 to –35 | 1.10 | | 12.3 | .014 |
| P–2 | U | –35 to –10 | 8.39 | | 2.0 | .017 |
| | P | –1 | | | 1.50 | |
| | | 0 to 10 I.V. injection 50 cc 20% NaPAH. | | | | |
| 1 | U | 24 to 58 | 3.31 | 3800 | 11.0 | .036 |
| | P | 28 | | 62.2 | 1.54 | |
| 2 | U | 58 to 75 | 4.61 | 1529 | 6.8 | .031 |
| | P | 69 | | 14.1 | 1.56 | |
| 3 | U | 75 to 97 | 6.13 | 739 | 4.4 | .027 |
| 4 | U | 97 to 118 | 8.00 | 254 | 3.0 | .024 |
| W.H. Dehydration. 11 yrs. S.A. 1.12 $M^2$. Wt. 30.9 kg. | | | | | | |
| P–1 | U | –116 to –86 | 0.38 | | 53.2 | .020 |
| P–2 | U | –86 to –59 | 0.31 | | 59.4 | .018 |
| P–3 | U | –59 to –23 | 0.28 | | 67.8 | .019 |
| | P | –1 | | | 1.61 | |
| | | 0 to 5 I.V. injection 35 cc 20% NaPAH. | | | | |
| 1 | U | 22 to 42 | 2.88 | 4560 | 19.4 | .056 |
| | P | 25 | | 54.3 | 1.54 | |
| 2 | U | 42 to 63 | 1.63 | 4622 | 28.7 | .047 |
| | P | 56 | | 13.6 | 1.52 | |
| 3 | U | 63 to 82 | 0.82 | 4114 | 46.4 | .038 |

affect the renal excretion of several substances,[2–4] an effect on phosphate excretion has not been previously described. This report concerns studies of phosphate excretion at various plasma PAH levels under conditions of both hydration and dehydration.

*Procedure and methods.* The studies were done on one female and 5 male patients, 9 to 12 years of age, who showed no evidence of renal disease. One child had slight albuminuria and hematuria associated with an episode of acute rheumatic fever three weeks previous to the experiment but subsequently has shown no evidence of renal disease. The subjects were in the post-absorptive state. Two subjects were deprived of water for 16 hours prior to the procedure. In these the PAH produced osmotic diuresis. In the other patients, high urine flows were maintained by water intake as is commonly done for renal clearance studies.

The general procedure of 4 experiments was as follows: After the collection of several preliminary urine specimens and one blood specimen, sufficient 20% NaPAH was injected intravenously in a 10 to 15 minute interval to produce plasma levels of 60 mg % or higher. Four to six urine specimens at 15 to 20 minute intervals and 2 or more blood specimens were then collected.

In 2 of the hydrated subjects, the procedure was somewhat modified as will be described later.

In blood and urine, PAH was determined by the method of Smith *et al.*[5] and phosphate by the method of Fiske and Subbarow.[6] Urine volumes were corrected to 1.73 $M^2$ for the calculation of minute phosphate excretion.

*Results.* The pertinent data of 2 representative experiments during hydration and dehydration are given in Table I. For all experiments, phosphate excretion during preliminary urine periods prior to the injection of NaPAH ranged from 0.002 to 0.020 mOsmols/min. In every case the rate of ex-

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[2] Selkurt, E. E., *Am. J. Physiol.*, 1944, **142**, 182.

[3] Beyer, K. H., Woodward, R., Peters, L., Verwey, W. F., and Mattis, P. A., *Science*, 1944, **100**, 107.

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[5] Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

[6] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

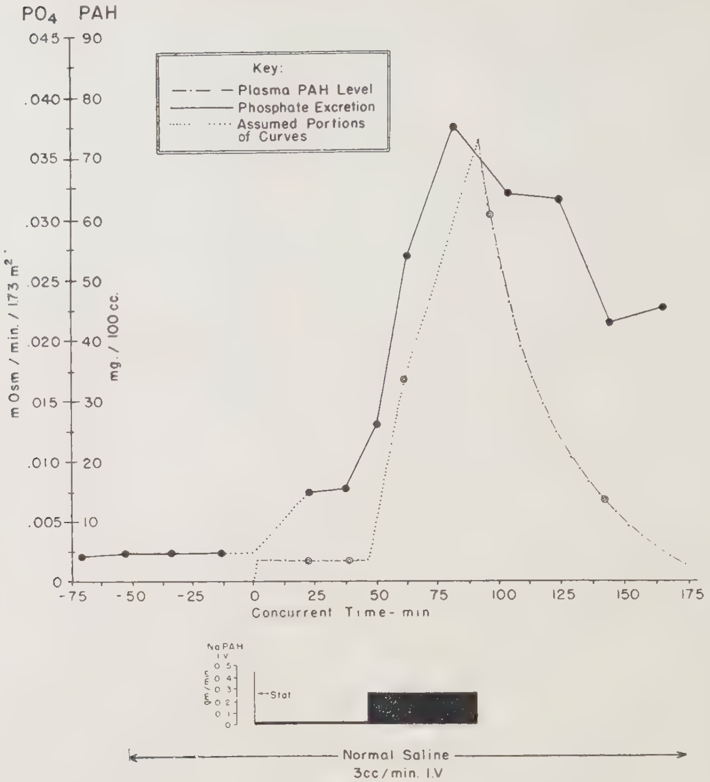

FIG. 1.

Phosphate excretion and plasma PAH level. See text for details.

cretion increased following the injection of NaPAH, regardless of the state of hydration of the subject. The maximum rate usually occurred during the first or second collection period following the rapid injection, not always coincident with the highest plasma PAH concentration. The maximum rate of phosphaturia ranged from 0.017 to 0.069 mOsmols/min., representing 2.3 to 16 fold increases over the respective rates during preliminary periods. After reaching a maximum, the excretion diminished at a rate coinciding roughly with the fall in plasma PAH concentration.

Plasma phosphate concentration did not change significantly during the course of the experiments. The phosphate level averaged 1.40 mOsmols/liter for six specimens taken prior to the injection of NaPAH and 1.46 mOsmols/liter for 19 specimens taken after the injection.

In Fig. 1 is presented one of two experiments so arranged as to observe phosphate excretion at constant low plasma PAH levels (3 to 5 mg %) and during the rise to and fall from higher concentrations. Throughout the experiment, with the exception of the first two preliminary periods, normal saline was injected intravenously at a constant rate of 3.0 cc/min. with sufficient NaPAH added at intervals to produce the plasma concentrations shown. The effect of PAH in increasing the rate of phosphate excretion is readily apparent.

*Discussion.* Under normal conditions extensive tubular reabsorption of phosphate takes place. Assuming a filtration rate of 120 cc/min., the amount of phosphate excreted during the preliminary periods of the experiments here presented averaged 7% of the amount filtered. After injection of NaPAH during the periods of greatest phosphaturia, assuming the same filtration rate, the phosphate excreted ranged from 9 to 45%, with an average of 26% of the amount filtered. The inhibition of tubular phosphate reabsorption by PAH is thus of considerable magnitude but is not sufficient to cause excretion to occur at the level of glomerular filtration.

It is worthy of note that the diminished tubular reabsorption of phosphate which occurs after injection of parathyroid hormone is of about the same order of magnitude. The data of Harrison and Harrison[7] on dogs show that after injection of parathyroid extract, 25 to 28% of the filtered phosphate was excreted. Filtration rates were determined by creatinine clearance in these experiments. In normal adults the ratio of excreted to filtered phosphate after parathyroid hormone injection can be calculated from the data of other workers, assuming a filtration rate of 120 cc/min. The 5 subjects of Ellsworth and Howard[8] excreted 11 to 41% of the phosphate filtered in the second and third hours after injection of 40 units of parathyroid hormone. Essentially the same percentage of phosphate excreted may be calculated from the data for the two subjects of Goadby and Stacey[9] after doses of 60 and 100 units of parathyroid extract.

Previous work has shown that the tubular transport of a number of substances may be depressed by PAH. The tubular reabsorption of ascorbic acid[2] and the tubular excretion of penicillin[3] are reduced by the presence of high plasma PAH levels. The inhibition of tubular transport of these substances may be so great that excretion occurs at the level of glomerular filtration. Glucose and PAH appear to inhibit reciprocally the tubular transfer of each other. In dogs the $T_m$ for glucose has been shown to be consistently depressed by high PAH levels.[4] In man, the opposite effect, an increased $T_m$ for glucose in the presence of high PAH levels, has been reported by Klopp, Young, and Taylor[10] in 2 out of 4 patients studied. An explanation of this unexpected finding may lie in the fact that in their experiments, the mannitol clearance, used as a measure of glomerular filtration rate, was found to be increased significantly with high PAH levels. It is possible that the augmentation of the mannitol clearance and consequently of the glucose $T_m$ was the result of an error in the determination of mannitol due to interference by PAH. Barker and Clark[11] have recently shown that in the method used by Klopp *et al.* (periodate-iodide-thiosulfate titrimetric procedure), PAH causes a large positive error, to the extent of 0.27 mg of mannitol per mg of PAH. The reciprocal effect, *i.e.*, inhibition of the transport of PAH by high plasma glucose levels has been consistently demonstrated in both dog and man.[4,10,12,13]

The nature of the inhibition of tubular transport can only be speculated upon. The inhibition of creatine reabsorption by glycine, alanine and glutamic acid[14,15] and of xylose reabsorption by glucose[16] has been attributed to competition for a common mechanism of limited capacity involving a single transport substance (B substance of Shannon[17]). Although such an explanation may be valid for structurally similar substances, it is difficult to conceive of one enzymatic or chemical mechanism sufficiently versatile to act in such a widely differing manner as to secrete PAH and penicillin or reabsorb ascorbic acid, glucose, and the phosphate ion. The circumstance that 4 of the 5 substances just mentioned are anions would suggest the existence of a tubular transport mechanism based on this property. However, the mutual inhibition of glucose and PAH speaks strongly against such an assumption. Another point, possibly contradicting the idea of a common mechanism, is the fact that one of the substances, phosphate, is an inorganic ion.

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[7] Harrison, H. E., and Harrison, H. C., *J. Clin. Invest.*, 1941, **20**, 47.

[8] Ellsworth, R., and Howard, J., *Bull. Johns Hopkins Hosp.*, 1934, **55**, 296.

[9] Goadby, H. K., and Stacey, R. S., *Biochem. J.*, 1934, **28**, 2092.

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[14] Pitts, R. F., *Am. J. Physiol.*, 1943, **140**, 156.

[15] Pitts, R. F., *Am. J. Physiol.*, 1944, **140**, 535.

[16] Shannon, J. A., *Am. J. Physiol.*, 1938, **122**, 775.

[17] Shannon, J. A., *Physiol. Revs.*, 1939, **19**, 63.

The inhibition of tubular transport by PAH may be more adequately explained by the hypothesis of a common energy source of limited capacity. Competition for the available energy by the activity of the mechanism for the transport of one solute would then result in diminished transport of another solute. Neither the locus nor the character of the energy limitation can be stated. Some of the possible mechanisms involved have been discussed by Selkurt[2] and by Houck.[4]

It should be emphasized that the inhibition of tubular transport of one substance by another may result in serious error when several renal functions are measured simultaneously.

*Summary.* The urinary excretion of phosphate is increased by the injection of sodium p-aminohippurate in man. The extent of the phosphaturia tends to vary directly with the plasma PAH level. At high PAH levels, the amount of phosphate excreted averages 26% of that filtered, a proportion comparable in magnitude to that reported after injection of parathyroid hormone.

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## 17180. Effect of Dietary Cholesterol on the Metabolic Rate of Hyperthyroid Rats.[*]

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It was recently observed that dietary cholesterol increases the survival time of rats fed toxic doses of thyroid hormone.[1,2] In an attempt to learn whether this protective action of cholesterol is correlated with a corresponding modification of the effects of thyroid on the metabolic rate, the influence of dietary cholesterol was investigated on the oxygen consumption of hyperthyroid rats.

*Methods.* Thirty-two male rats (USC strain), about 9 weeks of age, were distributed evenly among 4 groups which received the following diets *ad libitum*: I. This laboratory's stock diet, slightly modified to contain more vitamin B complex[‡] ("N"). II. Diet I, containing, in addition, 1 to 2% cholesterol[§] ("C"). III. Diet I, containing, in addition, 0.15% desiccated thyroid U.S.P.[§] ("T"). IV. Diet I, containing, in addition, both 0.15% thyroid and 1 to 2% cholesterol. ("T + C"). At first, cholesterol was fed at a level of 1%, but after 3 weeks, its concentration in the diet was raised to 2%. The cholesterol was dissolved in hot cottonseed oil, replacing a corresponding amount of oil in the diet. In order to increase cholesterol absorption, 0.25% bile salt[§] was added to all diets.[3]

The oxygen consumption was determined using a closed circuit apparatus provided with separate chambers for individual animals.[4] After repeated measurements during a preliminary training period of 5 weeks, feeding

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[*] These data are from a thesis presented by Louis E. Winebrenner to the Graduate School of the University of Southern California in partial fulfillment of the degree of Master of Science. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation. Paper No. 219 from the Department of Biochemistry and Nutrition.

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[1] Marx, W., Meserve, E. R., and Deuel, H. J., Jr., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 385.

[2] Ershoff, B. H., and Marx, W., *Exp. Med. and Surg.*, 1948, **6**, 145.

[‡] Whole wheat, 32.5%; oats, 32.75%; skimmed milk, 10%; alfalfa meal, 4%; yeast, Anheuser-Busch strain G, 9.5%; Wesson oil, 8%; fortified oil containing 1500 I.U. vitamin A/g and 160 I.U. vitamin $D_2$/g, 2%; sodium chloride, 0.5%; calcium carbonate, 0.5%; bile salt, 0.25%.

[§] Cholesterin c.p., Amend Drug and Chemical Co., New York City; desiccated thyroid, U.S.P., Armour and Co., Chicago, Ill.; sodium glycocholate (pure), City Chemical Corp., New York City.

[3] Schoenheimer, R., *Biochem. Z.*, 1924, **147**, 258.

TABLE I.
Effects of Dietary Thyroid and Cholesterol in Male Rats.
Figures are Average Oxygen Consumption and Standard Error.

| Group* | Thyroid fed | Cholesterol fed | At onset | After 3 wks | After 6 wks | After 9 wks |
|---|---|---|---|---|---|---|
| | | | ml oxygen/100 g body wt/hr | | | |
| N | — | — | 123 ± 4 | 106 ± 3 | 108 ± 4 | — |
| C | — | + | 121 ± 2 | 106 ± 3 | 105 ± 3 | — |
| T | + | — | 122 ± 2 | 166 ± 4 | 162 ± 6 | 160 ± 6 |
| T + C | + | + | 121 ± 4 | 157 ± 3 | 144 ± 3 | 144 ± 4 |

* Each group consisted of 8 rats.

TABLE II.
Effects of Dietary Thyroid and Cholesterol in Male Rats. Average Body Weight Gain, Food Intake, and Tissue Cholesterol Content.

| Group* | Thyroid fed | Cholesterol fed | Body wt gain in 9 wks and standard error, g | Food intake,† g/100 g body wt/day | Cholesterol content† | |
|---|---|---|---|---|---|---|
| | | | | | Plasma, mg/100 ml | Liver, % |
| N | — | — | 122 ± 5 | 5.7 | 53 | 0.21 |
| C | — | + | 109 ± 7 | 5.9 | 62 | 0.79 |
| T | + | — | 80 ± 7 | 7.1 | 49 | 0.24 |
| T + C | + | + | 72 ± 8 | 8.2 | 54 | 0.81 |

* Each group consisted of 8 rats.
† No standard errors are given, since, in each group, the samples were pooled in lots of 2 or 3.

of the experimental diets was begun, and the oxygen consumption was determined every 3 weeks. Body weights were recorded every 10 days, and the food consumption was measured at 2 day intervals during the seventh and eighth week.

After an experimental period of 9 weeks, the animals were autopsied, and weights and total cholesterol contents of various tissues were measured, the latter according to a modified Schoenheimer-Sperry-Chaney procedure.[5]

*Results.* The data on the determination of the oxygen consumption indicate that the administration of thyroid raised the metabolic rate by about 60% (Table I). The addition of cholesterol to the high thyroid diet caused a reduction of this value by about 10%, the difference being statistically significant ($P < 0.01$ for the 6 week period; $P =$ approximately 0.025 for the 9 week period). The metabolic rate of normal rats was not significantly modified by the addition of cholesterol to the diet.

Thyroid administration depressed the body weight gain and stimulated the animal's appetite, as was to be expected (Table II). The addition of cholesterol to the diet did not modify the food intake significantly, neither in normal nor in hyperthyroid rats. It is obvious, therefore, that the depressing effect of dietary cholesterol on the metabolic rate can not be attributed to a decrease in food intake.

Liver weights were found increased as a consequence of both thyroid and cholesterol administration, group T + C showing the highest values. Thyroid hormone, but not cholesterol, caused significant enlargement of the kidneys and adrenals also. These changes were to be expected.

Although total cholesterol concentrations of the plasma were not much modified, the level of liver cholesterol was markedly increased by cholesterol feeding, regardless of the thyroid content of the diet (Table II).

*Discussion.* Although cholesterol feeding significantly reduced the oxygen consumption of the hyperthyroid rats, the decrease amounted to only about 10% and was not sufficient for the metabolic rate to approach normal levels. The recently reported protective action of cholesterol as regards the survival time of thyrotoxic rats is much more pronounced;[1,2] it is questionable, therefore, whether this effect on the survival time is based on a mechanism related to the meta-

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[4] Mason, G. D., and Winzler, R. J., to be published. The authors are much obliged to Dr. Winzler for the privilege of using this apparatus.

[5] To be published.

bolic rate.

The results reported here may be related, however, to observations of Saegesser,[6] who reported a depression by cholesterol of thyroxine effects on the rate of metamorphosis of tadpoles, and on the oxygen requirement of rats in the oxygen deficiency test ("Sauerstoffmangelversuch") of Asher and Streuli.[7]

*Summary.* Dietary cholesterol significantly decreased the oxygen consumption of thyroid-fed rats, but the reduction was not sufficient for the metabolic rate to approach normal levels. This effect of cholesterol cannot be attributed to a depression of the food intake.

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[6] Saegesser, M., *Klin. Wochenschr.*, 1933, **12**, 672.

[7] Asher, L., and Streuli, H., *Biochem. Z.*, 1918, **87**, 359.

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## 17181. Calcification of Cell Cultures in the Presence of Embryo Juice and Mammalian Sera.

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Upon undertaking to learn if leprosy bacilli would multiply in cells cultured from leprous lesions, the mistake was made of trying to use the serum and chick embryo juice in proportions suitable for the cultivation of chick tissues. Following the first or second renewal of a liquid phase composed of human serum 25% and chick embryo juice 15%, the central explants or transplants became opaque to transmitted light and a brownish precipitate accumulated in the surrounding plasma. Once this happened a further renewal of the same type of medium caused a dense precipitate to appear among the growing cells, inhibiting growth and usually resulting in loss of the cultures. In the meantime the central fragments have acquired, by reflected light, a whitish color due to the accumulation of bone salts, and could be heard to crunch when cut with a knife. Though the phenomenon described may have been an extreme example of rapid calcification in cell cultures, similar difficulties have plagued other investigators. Earle[1] reported trouble with opaque plasma (in this case in areas removed from the colony sites) during the cultivation of Walker's rat carcinoma in horse serum and chick embryo juice. Gey and Gey[2] reported unfavorably on the use of chick embryo juice with human cord serum and later stated[3] that the difficulty was associated with turbidity in the plasma. Parker[4] reports that "these precipitates have been encountered in many laboratories and in every sort of culture. In Fischer's laboratory, in Berlin-Dahlem, where there was a lot of it at one time, the general feeling developed that it was a calcium compound and related in some way to the Tyrode's solution that was being used."

Since chick embryos were the only source of tissue extract available at Culion, the factors causing calcification were studied and means developed for avoiding or minimizing these difficulties in the presence of mammalian sera.

*Methods.* Cultures were prepared as previously described,[5] except that 2% embryo juice was used to transfer the 1.5 mm explants of human skin, leproma, or divided tissue culture to the 13 mm tubes containing one drop of chicken plasma 50%. After adding 0.5 ml of liquid phase and equilibration under 30 mm (4%) $CO_2$, the tubes were incubated in a slanting position at 34°C. Supernatant fluids were replaced each 7 to 14 days, *i.e.*, as the pH fell from 7.5 to 7.0. The plasma was patched at these intervals as required. All cultures were examined each week and, until their shape became irregular as they almost filled the plasma, measured along two diameters by means of an ocular micrometer. For the present purpose additional notes were made on the pH and the turbidity of the media, the occurrence of precipitate in the liquid phase, and on the opacity of the explants and of the plasma.

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* The greater part of the work was done in the Leonard Wood Memorial Laboratory, Culion, Philippines, 1941-43.

[1] Earle, W. R., *Am. J. Cancer*, 1931, **24**, 566.

[2] Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

[3] Gey, G. O., personal communication.

[4] Parker, R. C., personal communication.

[5] Hanks, J. H., *J. Cell. and Comp. Physiol.*, 1948, **31**, 235.

The chicken plasma *(CH)* contained 0.2% sodium citrate. Chick embryos were pressed through a Latapie grinder and extracted with 2 ml balanced salt solution (lacking $NaHCO_3$) per gram of embryo. These suspensions were spun immediately in the SP angle centrifuge for 30 minutes at 3000 RPM. The supernatants, after having been respun in the same way, were remarkably clear except for an upper stratum of lipoid. When adjusted to neutrality and buffered with 0.125% $NaHCO_3$, the concentration of these extracts became 30% ($EM_{30}$). The salt solution, Sol. A, (otherwise as before)[5] contained 0.2 g per liter of $CaCl_2$ and 0.06 g each of $Na_2HPO_4 \cdot 2H_2O$ and of $KH_2PO_4$. After adding to the autoclaved salt solution 10% by volume of sterile 1.4% $NaHCO_3$, the concentration of bicarbonate became 0.127%, of Ca 6.5 mg%, and of inorganic P 2.2 mg%. A similar *Sol. N* (no Ca or P) and other modifications will be mentioned in the text.

In the early work placental serum (PLS) was collected as available and preserved by Chryochem dehydration. When reconstituted, 0.1% glucose and 0.002% phenol red were included and the serum further diluted to 50% in the balanced salt solution. It was sterilized by pressure filtration (5% $CO_2$) through a Seitz filter. The Seitz pads were found to contribute Mg ions and alkaline radicals, and this method was replaced by aseptic collection of serum. The composition of fluid media will be indicated in code, *e.g.*, $PLS_{25}EM_{15}$ referring to placental serum 25%, embryo juice 15%.

*Chemical determination.* Both calcium and inorganic phosphate were determined in trichloracetic acid filtrates. Calcium was determined by Tisdall's modification[6] of Kramer and Tisdall's method and phosphorus by Brigg's[7] modification of the Bell and Doisy method. Values for Ca and inorganic P in mg% will be written as exponents. Ca x P will designate the product of these values. For example, Ca x P of the salt solution: $Ca^{6.5}$ x $P^{2.2}$ = 14.3.

*Experimental results.* After many early attempts to employ 15% chick embryo juice in the presence of 25% human serum, it became evident that several conditions influenced the calcification. These factors included: the age of the embryos employed, the proportion of embryo juice mixed with the serum, and different methods of preparing, storing, or treating the embryo juice.

*Age of Embryos.* Various batches of extract from pools of 10-day-incubated embryos differed remarkably in their ability to cause calcification. Since Philippine chickens hide their nests, overage eggs were unavoidable among the freshest obtainable. On one occasion marked calcification resulted from a single application of medium containing embryo juice from a pool of 5 embryos, one of which was perhaps 12 or 13 days actual age. From such observations it became apparent that calcification *in vitro* was related to the development of the calcifying mechanisms of the chick embryo. Data eventually collected on Ca and P values in relation to the age and weight of embryos (see Table I) revealed that the Ca x P of the embryo juice averaged 68.5 by the 11th day of incubation and reached approximately 100 by the 12th day. It will be noted that the typical Ca : P ratios at 11 days average 1:2.4 and *are inverted by comparison with mammalian ratios.*

Comparisons between extracts from 9- and 11-day embryos from the same lots of eggs revealed that the juice from the 11-day embryos (in $PLS_{25}EM_{15}$) usually induced calcification after second renewal of the medium while juice from the 9-day embryos permitted 3 or 4 renewals. 9-day extracts, however, were inferior to 11-day juices for growth stimulation and for continuous main-

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[6] Tisdall, F. F., *J. Biol. Chem.*, 1923, **56**, 439.

[7] Briggs, A. P., *J. Biol. Chem.*, 1922, **53**, 13.

TABLE I.
Effect of Age of Pooled Embryos on the Ca and P Values of Fresh $EM_{30}$.

| Pool No. | Incubation of eggs (days) | Avg wt* of embryos (g) | Ca (mg %) | P (mg %) | Ca x P | Ratio Ca : P |
|---|---|---|---|---|---|---|
| 1 | 8 | 1.0 | 4.0 | 7.4 | 29.6 | 1: 1.9 |
| 2 | 10 | 1.5 | 4.1 | 10.2 | 41.8 | 1: 2.5 |
| 3† | 11 | 1.9† | 6.2† | 8.7† | 53.9† | 1: 1.4† |
| 4 | 11 | 2.3 | 5.4 | 12.3 | 66.4 | 1: 2.3 |
| 5 | 11 | 2.4 | 5.2 | 13.1 | 68.1 | 1: 2.5 |
| 6 | 11 | 2.6 | 5.5 | 12.9 | 71.0 | 1: 2.3 |
| 7 | 12 | 3.0 | 7.0 | 14.9 | 104.2 | 1: 2.1 |
| Avg values for No. 4-6 (11-day Em) | | 2.4 | 5.4 | 12.8 | 68.5 | 1: 2.4 |

* These eggs were much smaller than American eggs.

† Unusual Ca and P values and not averaged with No. 4-6; the only 11-day EM studied which failed to cause calcification when used at 15% levels.

tenance of human fibroblasts. It was decided to continue the work with extracts prepared from 11-day embryos.

*Varying proportions of serum and embryo juice.* As may be seen in Table II, the use of varying proportions of 50% serum and 30% embryo juice in a series of experiments revealed that $PLS_{17}EM_{20}$ usually produced calcification after a second application (following one renewal): $PLS_{25}EM_{15}$ ordinarily permitted 3 applications; $PLS_{33}EM_{10}$ was tolerated for 4 or sometimes 5 applications, while $PLS_{42}EM_{5}$ could be employed during intervals up to 8 weeks. The latter medium was the only one which assured that long-term cultures would not be lost due to calcification during repeated transplantation. In it the explants or transplants sometimes turned opaque but the precipitate did not spread among the growing cells.

From the Ca and P data in the table it is apparent that the media with higher proportions of embryo juice contained P in excess of Ca. Furthermore, the higher Ca x P values occur where there is the least serum protein to stabilize these compounds in solution.[8] It was only when the embryo juice was reduced to 5% that Ca was appreciably in excess of P and the "potential" Ca x P could be expected to remain below 60.

Since P is liberated during incubation of the cultures, and both Ca and P are absorbed in the plasma and the explants, these potential values could not be determined without destruction and analysis of cultures being maintained for another purpose. Evidence that fresh embryo juice is saturated with respect to Ca and P and that active phosphatases liberate still more P during incubation will be presented in justification of the calculations employed.

*Embryo juice as a solution saturated with Ca and P.* Though the actual levels of Ca and P tended to vary in different lots of extract from 11-day embryos, these values were usually related inversely to each other and showed fairly good correlation between the Ca x P products and the average weights of the embryos employed. The relatively clear extracts were observed to become more opalescent or actually turbid during refrigeration. When incubated at 37° for a few days, they quickly passed through a cycle of opalescence, turbidity and sedimentation. In 9 extracts which were allowed to pass through this cycle for 3 to 5 days at 37° the average initial values of $Ca^{5.3} \times P^{12.6} = 66.8$ had changed to $Ca^{4.2} \times P^{16.1} = 67.6$ after incubation. These products indicate that the fresh embryo juice was saturated. The increase in P had been more than the indicated 28%, since some had been involved with the Ca precipitated.

Other evidence of saturation appeared upon trying to suspend fresh embryo pulp in salt solutions designed to lower or modify the levels of Ca and P. 4 batches of 11-day embryos were pulped without diluent. One

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[8] Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 699.

TABLE II.
Ca and P Values Upon Mixing Various Proportions of Placental Serum 50% with 11-day Chick Embryo Juice 30%, and Their Relation to Calcification of Tissue Cultures.

| Formula of medium % | Proportion of each component | Ca and P contributed by each<br>Ca mg % | <br>P mg % | C : P ratio | Initial Ca x P product of medium | Applications required to produce calcification | Potential Ca x P product of medium |
|---|---|---|---|---|---|---|---|
| PLS 50* | 6/6 | 9.0 | 5.0 | 1: 0.6 | (45.0) | | |
| EM 30* | 6/6 | 6.0 | 13.0 | 1: 2.2 | (78.0) | | |
| PLS 17 | 2/6 | 3.0 | 1.7 | | | | |
| EM 20 | 4/6 | 4.0 | 8.7 | 1: 1.5 | 72.8 | 2 | 79.3 |
| | | T 7.0 | 10.4 | | | | |
| PLS 25 | 3/6 | 4.5 | 2.5 | | | | |
| EM 15 | 3/6 | 3.0 | 6.5 | 1: 1.2 | 67.5 | 3 | 74.8 |
| | | T 7.5 | 9.0 | | | | |
| PLS 33 | 4/6 | 6.0 | 3.3 | | | | |
| EM 10 | 2/6 | 2.0 | 4.3 | 1: 1.0 | 60.8 | 4 or 5 | 67.5 |
| | | T 8.0 | 7.6 | | | | |
| PLS 42 | 5/6 | 7.5 | 4.2 | | | | |
| EM 5 | 1/6 | 1.0 | 2.2 | 1: 0.8 | 54.4 | 6 to 8 | 58.1 |
| | | T 8.5 | 6.4 | | | | |

* Composition of ingredients used to prepare the medium.
Method of calculating initial Ca : P and Ca x P shown in detail.
T = Total Ca and P upon making mixtures. Potential Ca x P calculated in similar manner, using the Ca and P values of $EM_{30}$ which has been incubated for one week, *i.e.*, $Ca^{4.6}$, $P^{16.9}$.

half of the pulp was suspended in the usual *Sol A* and the other half in *Sol N* (no Ca or P). The extracts were centrifuged immediately. The extracts in *Sol A* averaged $Ca^{5.3}$ x $P^{13}$ = 68.9, while those in *Sol N* averaged $Ca^{4.1}$ x $P^{16.1}$ = 66. In this brief interval the pulp had practically saturated *Sol N* with these elements, P exceeding Ca by approximately 4 times. When employed in serum media of the type illustrated in Table II, the extracts in *Sol N* produced more rapid calcification than those in *Sol A*. Calculation shows that this was to have been expected from the high Ca x P values produced upon mixing the high Ca of the serum and salt solutions with tissue extracts having P values around 16.

Correct Ca and P ratios (but high products) could be produced in fresh extracts by diluting the pulp in balanced salt solution containing elevated Ca and no P. Upon incubation, however, these extracts precipitated excessively. Calculation indicated that this modification was impractical, since each mg% of additional P liberated by phosphatases must now be multiplied by a higher initial level of Ca.

Since P is dialyzable, correct values and ratios for Ca and P should be obtained by dialysis of embryo juice. A fresh embryo extract containing $P^{10.7}$ was diluted with 2 volumes of balanced salt solution containing $Ca^7P^0$, to produce an anticipated value of $Ca^{6.5}$ and $P^{3.6}$. Upon being rapidly concentrated to the original volume in a pressure dialyzer modified after Simms,[9] the first half of the filtrate contained 3.5 mg% of P, the second half 3.6, and the residue in the bag 3.7 mg% of P. Though this procedure can produce embryo juice of any desired P value, it is not practical as a routine and also washes out small molecules which are important to nutrition.

*Pasteurization to inactivate phosphatases.* Inactivation of phosphatases by pasteurizing the embryo juice at 60° for 30 minutes, which causes some coagulation of protein, reduced the initial P values by 1-2 mg% and the Ca x P products by approximately 6 to 12. The P values did not rise on incubation of this juice alone and rose only slowly during the incubation with serum, presumably due to serum phosphatase. These extracts, when used in $PLS_{25}EMP_{15}$ induced good growth and permitted continuous maintenance of several series of cultures in which all of the control cultures in unheated embryo juice were lost by calcification. The pasteurization of embryo juice has previously been shown to inhibit the liberation of free lipoid and to promote greater longevity of chick embryo cultures than is possible with fresh embryo juice.[5]

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[9] Simms, H. S., and Stillman, N. P., *J. Gen. Physiol.*, 1937, III, **20**, 649.

*Calcification studies in plasma blanks with 4 types of serum.* Though growing cell colonies obviously play a role in phosphate liberation and CaP deposition near the central explants, blank tests in the plasma base with serum-embryo juice mixtures renewed at weekly intervals provided a convenient method for comparing different degrees of supersaturation, for example in systems containing serum from different animal species. These experiments were undertaken because Carrel had warned against the opacity which develops promptly if rabbit serum is employed in tissue cultures, because our attempts to substitute bovine for human serum had always increased calcification, and because the embryo juices referred to in this study had not been causing calcification in chick embryo tissue cultures.

The data in Table III summarize the observations on 3 batches of embryo juice in combination with 4 animal sera. Though the human and bovine sera gave comparable results in 2 of the trials, it is evident from the summarized scores, together with the experience in tissue cultures, that the 4 sera may be arranged as follows with respect to calcification: rabbit> bovine> human> chicken.

TABLE III.
Calcifying Tendency of Serum from 3 Mammalian Species and from Chickens, as Revealed in Plasma Blanks.

| Embryo juice No. | Opacity in plasma following weekly renewal with serum 25% and embryo juice 15% (sera) | | | |
|---|---|---|---|---|
| | Rabbit | Bovine | Human | Chicken |
| 63 | 034 | 012 | 012 | 000 |
| 67 | 044 | 023 | 023 | 000 |
| 70 | 144 | 023 | 002 | 001 |
| Total scores* | 222 | 58 | 37 | 1 |

* The 3 values for each serum represent the opacity rating on a plus scale at the end of the first, second and third weeks of the experiment. For example, 034 indicates no opacity after 1 week, 3+ opacity after 2 weeks and 4+ after 3 weeks. To illustrate the difference between the sera, these values are simply added as a total "score."

During these tests in the absence of cell colonies it became evident that a slowly increasing opalescence of the supernatant media containing mammalian sera provided the most favorable circumstance for calcification of the plasma layer, but that chicken serum media could become highly opalescent without inducing calcification. When incubated in plain glass tubes for several weeks, both rabbit and chicken serum media liberated more free lipoid (which appeared as a scum on the surface) than the bovine or human sera. The relation of free lipoid to lipoid accumulation in cells has been emphasized elsewhere.[5]

*Preparation of media with acceptable Ca and P content.* The data in Table IV provide a basis for estimating the total Ca and P content of media composed of human placental serum and chick or beef embryo juice. The ingredients differ from those used at Culion. The salt solution (prior to the addition of buffer) contains only 5 mg% of Ca, the approximate concentration of diffusible Ca in human serum. When mixed with any proportion of serum it provides ionized Ca levels similar to those in body fluids of corresponding protein content. Both chick and beef embryo juice should be prepared at 50% concentrations. Dilute extracts will tend to be saturated with Ca and P. If such extracts comprise a large portion of the final medium, it is impossible to dilute or modify their inorganic constituents to appropriate levels. When embryo juice is employed in concentrations greater than 5%, it is not necessary or desirable to incorporate P in the salt solution used for diluting the medium.

Since 10:1 ratios of serum and embryo juice seem to be optimal for good growth and quality of mammalian cells, the examples in the table are based on these ratios. With formulae such as $PLS_{60}EM_{6}$ the initial Ca x P may be nearly 55, while the "po-

TABLE IV.
Data for Estimation of Initial Ca and P Products of Media Which Combine Human Serum with Chick or Beef Embryo Juice.

| | Placental serum | | | $EM_{50}$, chick (11-day embryos) | | | $EM_{50}$, beef* (4-inch embryos) | | | Balanced salt solution | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | Ca | P | % | Ca | P | % | Ca | P | % | Ca | P |
| 1. | 100 | 12.0 | 5.5 | 50 | 4.3 | 13.9 | 50 | 10.5 | 12.0 | 100 | 5.0 | 2.4 |
| 2. | | | | 15† | 1.3 | 4.2 | 15† | 3.2 | 3.6 | | | |
| | | | | 10 | 0.9 | 2.8 | 10 | 2.1 | 2.4 | | | |
| | 60 | 7.2 | 3.3 | 6† | 0.5 | 1.7 | 6† | 1.3 | 1.4 | 28 | 1.4 | 0.7 |
| | 50 | 6.0 | 2.8 | 5 | 0.4 | 1.4 | 5 | 1.1 | 1.2 | 40 | 2.0 | 1.0 |
| | 40 | 4.8 | 2.2 | 4 | 0.3 | 1.1 | 4 | 0.8 | 1.0 | 52 | 2.6 | 1.3 |
| | 30 | 3.6 | 1.7 | 3 | 0.3 | 0.8 | 3 | 0.6 | 0.7 | 64 | 3.2 | 1.5 |
| | 20 | 2.4 | 1.1 | 2 | 0.2 | 0.6 | 2 | 0.4 | 0.5 | 76 | 3.8 | 1.8 |
| 3. | 10 | 1.2 | 0.6 | 1 | 0.1 | 0.3 | 1 | 0.2 | 0.2 | 88 | 4.4 | 2.1 |

1. Ca and P of constituents employed.

2. These concentrations of embryo juice, though satisfactory with chicken serum, are not recommended in the presence of mammalian sera.

3. If using 10:1 ratios of serum and embryo juice simply add the values on appropriate horizontal line, using data for the embryo juice employed. Otherwise add the values for the actual concentrations being used.

Example

| | % | Ca | P | Ca x P |
|---|---|---|---|---|
| $PLS_{40}$ | 40 | 4.8 | 2.2 | |
| $EM_4$ (beef) | 8 | 0.8 | 1.0 | |
| Salt sol | 52 | 2.6 | 1.3 | |
| | | 8.2 x | 4.5 = | 36.9 |

* Data from Dr. Geo. O. Gey, extract prepared in salt solution with $Ca^{9.9}$ and $P^{3.6}$.

† Values take into account the fact that 50% extracts occupy twice the volume indicated by the percentage concentrations.

tential" Ca x P will exceed 60. The suitability of such formulae presumably depends on the fact that the increased concentration of serum protein raises the levels of Ca x P which can be kept in solution.[8]

*Discussion.* The calcification of tissue cultures in mammalian sera as here described should not be confused with the Liesegang rings of calcium salts which were observed in "aerobic" chick embryo cultures by Hueper and Russell[10] and others to whom they refer. Calcification does not occur in chick cell cultures at appropriate pH, even in high concentrations of embryo juice and in the presence of 40% oxygen. The phenomenon of Hueper and Russell[10] must be attributed to excessive alkalinization in cotton stoppered vessels.

Since mixtures of embryo juice and chicken serum may become highly opalescent without inducing calcification, the difference between chicken serum and the mammalian sera may be related in part to the unusual capacity of chicken serum to carry high Ca and P values.[11] Further indication of differences between chick and mammalian systems is afforded by the observations of Fell and Robinson[12] on the resistance of the chick femur to calcification.

The calcification of mammalian tissue cultures in the presence of mammalian sera under similar circumstances is explained primarily by the high P values contributed by embryo juice and by the lower levels of bone salt required for saturation, while the differences between the 3 mammalian sera are doubtless related to differences in the natural levels of total and diffusible calcium.[13]

It would appear that cultivated mammalian tissues may differ in their phosphatase activity *in vitro*. With rat tissues in the media here described the plasma tended to remain clear in the region of the cell colonies and to become opaque elsewhere. Similar observations have been recorded by Earle.[1]

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[10] Hueper, W. C., and Russell, M. A., *Am. J. Med. Sciences*, 1933, **186**, 383.

[11] Greenberg, D. M., Lawson, C. E., Pearson, P. B., and Burmester, B. R., *Poultry Science*, 1936, **15**, 483.

[12] Fell, H. B., and Robinson, R., *Biochem. J.*, 1934, **28**, 2243.

[13] Schmidt, C. A., and Greenberg, D. M., *Physiol. Rev.*, 1935, **15**, 297.

In the presence of human tissues the freshness and potential phosphatase activity of the embryo juice influenced the type of calcification. If embryo juice was mixed with undiluted serum during refrigeration, the precipitation cycle and liberation of P was inhibited. The media subsequently prepared possessed excellent growth stimulating properties and the cell colonies appeared to play a role in phosphatase activation, since calcification centered around the transplants and among the growing cells. If, on the other hand, media were prepared from incubated juices which had passed through the precipitation cycle, the growth promoting properties were greatly diminished and calcification occurred diffusely throughout the plasma as though resulting from ionic exchange and simple supersaturation.

The present suggestions concerning the desirability of keeping the Ca x P products below 60 and the serum and embryo juice ratios at 10:1 serve only as an approximation of permissible thresholds under average circumstances. The work of Robinson and coworkers[8] reveals the manner in which protein, glucose, magnesium and the serum from different animal species influences the *in vitro* calcification of cartilage. From these and other data on the solubility of Ca and P in body fluids,[14,15] it is apparent that the permissible Ca x P will be related to the concentration of serum protein in the medium.

Several precautions should be borne in mind during the preparation of embryo juices and of serum. These include: (1) the use of 10- or 11-day chick embryos and exclusion of any which appear over-age; (2) the selection of beef embryos which do not exceed 4 inches in length; (3) the preparation and storage of extracts at 50% concentrations so that the final dilution will permit modification of the Ca and P to desired levels; (4) the maintenance of the lowest practical temperatures during extraction and centrifugation; (5) storage of the extracts in a frozen state. The loss of protein during pasteurization of 50% juices is excessive. If this procedure is employed to inactivate phosphatases, the juice should be diluted with an equal volume of salt solution just prior to heating. Hemolysis of red cells during serum production must be expected to elevate the P values[7] while filter pads which liberate magnesium, alkali, or other inorganic radicals should be avoided.

When growth inhibition or plasma opacity are shown to be due to calcification (as evidenced by darkening of the central transplants, by data on the Ca and P values of the medium, or by solubility of the opacity in N/10 HCl) the interim steps which should be taken are: (a) rapid transplantation with exclusion of the central portion of the colony or (b) tryptic digestion and removal of the opaque plasma if the colonies are to be retained in the original vessels. Re-evaluation and correction of the inorganic composition of the medium is necessary to prevent recurrence of the difficulty.

*Summary and conclusions.* 1. Chick embryo juice, in concentrations suitable for chick cell cultures, induces calcification of the cell colonies and plasma in mammalian cell cultures.

2. Fresh embryo pulp and extract are saturated with Ca and P. Ca and particularly P increase with age of the embryos, their ratios being 1:2.4 at 11 days. Upon incubation the P increases an additional 30%.

3. Pasteurization of embryo extracts inactivates the phosphatases and prevents calcification in cell cultures receiving high concentrations of embryo juice.

4. Calcification of cultures in the presence of mammalian sera is due to combining the Ca of serum and salt solution with the high levels of P in embryo juice, to the further liberation of P during incubation, and to the limited Ca x P products held in solution by mammalian sera.

5. The calcifying propensity of chicken serum is much less than that of 3 mammalian sera, which may be arranged as follows: human$<$bovine$<$rabbit.

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[14] Peters, J. P., and Eiserson, L., *J. Biol. Chem.*, 1929, **84**, 155.

[15] McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.